# EXPRESSION OF EPITHELIAL AND NEUROENDOCRINE MARKERS WITHIN CANINE APOCRINE GLAND ANAL SAC ADENOCARCINOMA (AGASACA) AND THE RELATIONSHIP TO PERIPHERAL HYPERCALCEMIA AND PARATHYROID HORMONE-RELATED PROTEIN (PTHrP) EXPRESSION

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Abstract

Apocrine gland anal sac adenocarcinoma (AGASACA) of the dog is a frequently invasive and metastatic tumor and is commonly associated with peripheral hypercalcemia due to circulating elevated parathyroid hormone-related protein (PTHrP). As the tumor name implies, this neoplasm is attributed to apocrine gland origin, and special staining is not typically performed for additional investigation. Few studies have pursued additional characterization of these masses and identified evidence of neuroendocrine differentiation within some canine anal gland tumors. At this time, however, the significance of this finding is unknown, though additional examination may reveal relationships between neuroendocrine differentiation and the occurrence of peripheral hypercalcemia, PTHrP expression (a frequent underlying cause of peripheral hypercalcemia), metastasis at time of diagnosis, and/or patient survival time.

The first objective of this study was to validate a PTHrP antibody for use in canine histology sections of AGASACA tumors. Secondly, we set out to determine if tumors that display neuroendocrine differentiation more commonly express PTHrP (a frequent underlying cause of peripheral hypercalcemia). A subset of canine anal sac apocrine gland tumors was retroactively evaluated via immunohistochemistry to determine tissue differentiation, histologic tumor pattern, and PTHrP expression. The third objective was to determine if a correlation between tumor PTHrP expression and peripheral hypercalcemia existed. The presence or absence of peripheral hypercalcemia was recorded by retrospective review of medical records for all patients within the study. Lastly, the relationships between histologic pattern and neuroendocrine differentiation, PTHrP expression, and peripheral hypercalcemia were evaluated. Tumor patterns were recorded during microscopic evaluation and the tumors were separated into one of seven groups based on their specific pattern.

There was no significant relationship between hypercalcemia and histologic tumor pattern. Results did show a significant correlation between hypercalcemia and positive PTHrP expression compared to absence of PTHrP expression. No statistical analysis was performed regarding neuroendocrine differentiation as no tumors showed positive reactivity.

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LIST OF ABBREVIATIONS

| | |
|---|---|
| ACTH | Adrenocorticotropic hormone |
| AGASACA(s) | Apocrine gland anal sac adenocarcinoma(s) |
| APUD | Amine precursor uptake and decarboxylase |
| CgA | Chromogranin A |
| DNES | Diffuse neuroendocrine system |
| GCDFP-15 | Gross cystic disease fluid protein-15 |
| H&E | Hematoxylin and eosin |
| HHoM | Humoral hypercalcemia of malignancy |
| iCa | Ionized calcium |
| IHC | Immunohistochemistry |
| mg/dL | Milligrams per deciliter |
| mmol/L | Millimoles per liter |
| MST(s) | Median survival time(s) |
| NCBI | National Center of Biotechnology Information |
| NEC(s) | Neuroendocrine cell(s) |
| NET(s) | Neuroendocrine tumor(s) |
| PNS(s) | Paraneoplastic syndrome(s) |
| PTH | Parathyroid hormone |
| PTHrP | Parathyroid hormone-related protein |
| RANK | Receptor activator NF-κB |
| RANKL | Receptor activator NF-κB ligand |
| SYN | Synaptophysin |

# CHAPTER 1

## INTRODUCTION

Neoplasms associated with the canine anal glands may arise and are known as apocrine gland anal sac adenocarcinoma (AGASACA) of the dog; this is a frequently invasive and metastatic tumor, commonly spreading to regional lymph nodes[1,2,3,4,5]. As the tumor name implies, this neoplasm is attributed to apocrine gland origin, and special staining is not typically performed for additional investigation with routine histopathologic evaluation. Histologically, these tumors can display variable morphologic patterns and few studies have pursued additional characterization of these masses; however, there is evidence of neuroendocrine differentiation within canine anal gland tumors, a finding of uncertain clinical significance[5,6,7].

At presentation, affected patients may present with a range of clinical signs; however, in some instances, the neoplasm is an incidental finding on a routine rectal examination. Treatment options for AGASACA include surgical removal of the mass, variable radiation doses, and chemotherapy. Treatment is determined on a case-by-case basis, with some patients requiring only surgery and others benefiting from a combination of treatment modalities[8]. Canine AGASACA is locally invasive and has a high rate of metastasis[1,3]. Median survival time (MST) for this neoplasm is highly variable depending on the report and characteristics of included cases, but some animals have a long survival time with care.

Hypercalcemia, due to elevated parathyroid hormone-related protein (PTHrP) circulating in peripheral blood, has been reported to occur in 27% to 51% of AGASACA patients and is considered a negative prognostic factor[1,6]. Calcium homeostasis is normally tightly regulated within the body, in large part due to the action of the parathyroid gland. Dogs with AGASACA and hypercalcemia have been found to have substantially elevated PTHrP concentrations, while

normocalcemic dogs with AGASACA had undetectable PTHrP concentrations[9]. Parathyroid hormone-related protein can alter serum calcium concentrations in several ways. Disruptions in the normal bone environment due to metastatic disease can also lead to alterations in calcium concentrations within the body. However, while metastasis of AGASACA is common, it rarely metastasizes to bone; rather, metastasis usually occurs first at the draining lymph nodes, later progressing to the liver and spleen[1].

AGASACAs are intriguing from a clinicopathologic perspective because they display microscopic characteristics similar to those observed in cytology preparations of tumors of neuroendocrine origin (ex. insulinoma). The cells within these tumors are round to cuboidal to columnar, small to medium in size with pale, eosinophilic, slightly granular cytoplasm, and commonly have indistinct cell borders. Nuclei are round and medium-sized, one to two small nucleoli are usually present, and the cells are commonly arranged in various patterns[3,10]. Despite the unusual cytologic appearance, histopathologically, apocrine glandular tissue is the reported origin for canine AGASACA. The neoplastic tissue does not present a diagnostic challenge with routine hematoxylin and eosin (H&E) stain, as AGASACA tumors have distinctly different histomorphologic features from other common tumors found in this area[10,11]. Interestingly, several studies have found evidence of neuroendocrine differentiation within AGASACAs[5,6,7]. One study described the immunohistochemical staining properties of canine AGASACA and found positive-staining neuroendocrine cells in limited numbers throughout the 8 tumors evaluated[5]. Neuroendocrine differentiated cells within anal gland tumor tissue may play a clinically important role in tumor biology and/or disease progression.

Another study evaluated histologic growth patterns (solid, rosette, tubular, mixed) and neuroendocrine differentiation of AGASACA to assess for relationships to clinical outcome[7].

Links were not identified, but case numbers in the evaluated groups were low. In review of the canine AGASACA literature, the author was unable to identify studies with large case numbers that assessed for clinical differences between tumors that display neuroendocrine differentiation and those that do not. In particular, it is unknown whether tumors that display neuroendocrine differentiation more commonly express PTHrP and are associated with peripheral hypercalcemia.

# CHAPTER 2

# LITERATURE REVIEW

## Apocrine Gland Adenocarcinoma of the Anal Sac

Assorted glands are found within the skin, including sebaceous and modified sebaceous glands, apocrine and modified apocrine glands, and merocrine glands[12]. Many of these same glands are found around the perianal region of the dog, with the addition of the circumanal (hepatoid) glands and apocrine glands of the anal sac[6,11,13]. Canine anal sacs are two structures located laterally to the anus between the muscles of the sphincter ani externus and internus. They are composed of connective tissue, apocrine glands, sebaceous glands, and low numbers of neuroendocrine cells, with a lining of stratified squamous epithelium[5,14]. Apocrine cells are cohesive and glandular epithelial cells that are distributed throughout the hairy skin of most mammals, including the dog. Depending on their location, these cells excrete their product via exocytosis or apocrine secretion[15,16]. The anal sac apocrine glands are tubular mural glands that are distinct from the merocrine anal glands within this region[17]. In conjunction with sebaceous glands in this area, the apocrine glands fill the anal sacs with secretory products[11]. This serous to viscous fluid is light beige to dark brown, sometimes granular, and contains variable numbers of desquamated epithelial cells, local bacteria, yeast, and rarely neutrophils and mononuclear cells[14,18,19]. Anal sac and glandular function are thought to be related to social and territorial interactions, though a defensive mechanism, similar to that of the skunk, is also considered possible[14].

Tumors in the canine perineum are quite common and, given the variety of glands present, an assortment of tumors can be found[11]. These include perianal gland tumors and two

neoplasms that can arise from the apocrine glands of the anal sac—benign neoplasm, the anal sac gland adenoma, and the malignant form, apocrine gland adenocarcinoma of the anal sac[10,12]. These tumors cannot be differentiated from one another grossly and histopathology is required to differentiate the somewhat common AGASACA from the exceedingly rare anal sac gland adenoma[12]. AGASACA can occur unilaterally or bilaterally (up to 10% of cases), but the mechanisms driving bilateral AGASACA formation are unclear[20]. Currently, there is no single underlying or predisposing cause; however, recurrent anal sac impaction and infection have been noted in some patients[20].

A sex predilection towards intact female dogs was initially described in the literature, but more recent studies suggest a possible increased incidence in neutered male dogs[10,12,17,21]. Data describing breed predispositions are lacking overall, but Polton et al. found that English cocker spaniels, Cavalier King Charles spaniels, and springer spaniels have 7.3:1, 2.6:1, and 2.2:1 averaged odds for developing AGASACA.

Clinical signs of AGASACA are variable, though several studies indicate that perianal swelling and tenesmus are most common[2,3,11]. Polyuria, polydipsia, scooting, tumor bleeding, and hind limb weakness are also notable clinical findings associated with AGASACA[1,2,3,10,11]. Still, some patients display no clinical signs and tumor size can be relatively small (average 9cm$^2$) at the time of diagnosis; consequently, this aggressive neoplasm is an incidental finding in 39-50% of cases[2,20]. In addition to difficult detection, metastasis to the sacral, internal iliac, and medial iliac lymph nodes tends to occur early in the disease process[2,4,5]. Many studies found greater than 50% of tumors (range 36-96%) had evidence of metastatic disease at the time of diagnosis, and diagnostic imaging appears to be relatively insensitive at perceiving metastasis[2,3,4,12]. Overall, diagnosis of AGASACA should include full hematology and serum

biochemical analysis, abdominal ultrasound, and computed tomography[20]. Cytologic evaluation of enlarged lymph nodes may also prove helpful, as it is highly sensitive for identification of disease, but this may not be true for microscopic metastatic disease within normal-sized lymph nodes[4].

Cytologically, the epithelial apocrine cells of AGASACA lack distinct borders; additionally, acinar-like structures are commonly noted. These features can impart a neuroendocrine appearance on cytology, resembling aspirates of endocrine tissues such as thyroid C cell tumors, parathyroid tumors, insulinomas, chemodectomas, pheochromocytomas, and carcinoids[22]. This contrasts with the appearance typical of other epithelial glandular tissues, such as salivary tissue. By way of example, cytologic preparations of salivary epithelium contain a uniform secretory epithelial population of primarily cohesive, rounded or polygonal cells with distinct borders, eccentric, deeply basophilic nuclei, and clear, vacuolated to foamy cytoplasm[23].

Histologically, AGASACA is made up of neoplastic epithelial apocrine cells separated by fine connective tissue strands and arranged in distinctive patterns: solid, rosette, tubular, and mixed[10]. Capillaries and pseudo-rosette structures are abundant[17]. Suzuki et al. found that solid-type and mixed-type tumors with solid components had poorer clinical outcomes than other histologic types. With H&E stain, the neoplastic cells of AGASACA are small to medium sized with pale eosinophilic, slightly granular cytoplasm and variably distinct cytoplasmic borders[10]. Nuclei are medium-sized and round with slight anisokaryosis and one or more small nucleoli[10]. Mitotic figures, stromal invasion, and metastasis are frequently seen[5,10].

Keratin-like proteins or cytokeratins are structural components of the cellular cytoskeleton that are characteristic of epithelial cells[24]. Vos et al. found positive cytokeratin 5, 7, 8, 18, and 19 activity in anal sac glands of dogs with immunohistochemistry. AGASACA tumors

were also positive for cytokeratin 7, 10, and 18, with variable staining for cytokeratin 14, 15, 17, and 19; these findings supported the tissue of origin of AGASACA tumors.

Treatment plans for patients diagnosed with AGASACA are created on a case-by-case basis but are generally based around surgical resection of the primary mass and enlarged lymph nodes, as studies have demonstrated that surgery seems to improve MST[2,25]. A study by Williams et al. asserts that patients have a 37% chance of surviving 2+ years and that dogs receiving surgery have a survival advantage, no matter what other adjunctive therapies are used. Some combination of radiation therapy or chemotherapy (carboplatin, melphalan, mitoxantrone, and cyclophosphamide) may also be included or used as a standalone treatment[2,4]. Bisphosphonates and saline hydration have been advised as treatments for hypercalcemia of malignancy[26].

Treatment complications vary depending on the chosen treatment modality. Infection, hypocalcemia, sudden death from anesthesia, fecal incontinence, wound dehiscence, perianal fistula, tenesmus, and complications due to radiation or chemotherapy can occur and reports of overall surgical complication rate range from 0-20%[2,4]. Negative prognostic indicators include masses that are $\geq 10cm^2$, lack of surgical intervention, presence of metastasis, and hypercalcemia[1,2,20,26].

Survival times are strikingly inconsistent throughout the literature, with studies reporting MSTs of 6-8 months, 18 months, 20 months, 29 months, and 41 months[1,2,25,27,28]. This is most likely due to many disease factors such as tumor size, early or late tumor detection, presence of metastatic disease, peripheral hypercalcemia (average MST of 256 days for hypercalcemic patients and 584 days for patients without hypercalcemia), and treatment plan[1,2,27].

Calcium and the Endocrine System

Calcium plays a large and varied role in many body systems, from skeletal support, blood coagulation, muscle contraction, hormone secretion, stabilization of sodium channels and cell membranes, and cell growth and division[29,30]. Complexed hydroxyapatite represents approximately 99% of the body's calcium supply. The remaining 0.9% and 0.1% are present intracellularly and extracellularly in other forms[29,31]. Total serum calcium consists of three fractions: roughly 50% biologically active ionized calcium, 40% protein-bound calcium, and 10% complex calcium in the form of bicarbonate, citrate, phosphate, lactate, and oxalate[31,32]. Total calcium is generally reported in milligrams per deciliter (mg/dL) or millimoles per liter (mmol/L), and the range in serum total calcium values may be wide within groups of normal dogs[32]. Ionized calcium is considered a more accurate measure of hypercalcemia because it only reports the active form of calcium[31,32,33,34]. As previously mentioned, calcium has many important and varied roles within the body, so it is of no surprise that even small increases in biologically active serum calcium concentrations above normal can have variable physiologic consequences and negative multisystemic impacts[30,31,32,33].

Calcium homeostasis is tightly regulated by parathyroid hormone (PTH), 1,25-dihydroxycholecalciferol (calcitriol), and calcitonin[31,33]. Serum calcium levels are maintained by the chief cells of the parathyroid gland. These cells synthesize and secrete PTH to maintain an appropriate serum calcium concentration at the level of the kidney and bone[34,35]. PTH acts on the PTH1 receptor at the surface of renal tubular epithelial cells, osteoblasts (bone producing cells), osteoclasts (bone resorbing cells), and bone stromal cells to promote calcium resorption[34,35]. It also promotes urinary phosphorus excretion, triggers activation of calcitriol, and indirectly increases intestinal absorption of dietary calcium[31]. Upregulation of the receptor activator of

nuclear factor κ-B ligand (RANKL) on the surface of osteoblasts also occurs. Osteoblasts then secrete RANKL that binds to receptor activator of nuclear factor κ-β (RANK) on osteoclasts, and several pathways stimulate osteoclast differentiation, activation, and survival, bone remodeling, and release of calcium into circulation[34,35,36].

Vitamin D (as vitamin $D_2$ and $D_3$) is directly absorbed within the intestinal tract and transported to the liver. It is then hydroxylated to 25-hydroxycholecalciferol (calcidiol) and further hydroxylated to the biologically active form of vitamin D (calcitriol) in the kidney by renal 1α-hydroxylase (activated by PTH)[31,34,35]. Calcitriol is responsible for the daily fluctuations in serum calcium. It acts in a fashion similar to PTH, promoting bone resorption, release of calcium into circulation, and renal reabsorption of calcium. Unlike PTH, calcitriol also promotes renal and intestinal reabsorption of phosphorus[31]. Another player in calcium homeostasis is calcitonin, which is produced by the C cells of the thyroid gland in response to increased calcium levels. Calcitonin, like PTH and calcitriol, acts on bone; however, it acts to decrease blood calcium levels by interfering with osteoclast-mediated resorption and increases calciuresis[31].

Thus, disturbances in calcium levels leading to hypercalcemia can cause an array of alterations in the body including polyuria, polydipsia, vomiting, inappetence, weight loss, paresis, skeletal pain and fractures[,37]. In the dog, hypercalcemia is defined as a fasting serum total calcium or ionized calcium greater than 12mg/dL or 5.81mg/dL respectively[32,37]. The endocrine system, of which certain parts play large roles in calcium regulation, is generally thought to include the pituitary gland, thyroid gland, parathyroid gland, adrenal gland, pancreas, and a few other organs. These organs regulate various crucial functions within the body such as metabolism, stress, development, and reproduction[38]. Cells within the endocrine system secrete peptides, amines, or steroids into circulation to maintain homeostasis within the body[39]. Serum

calcium concentration is mainly regulated PTH, vitamin D metabolites, and calcitonin, and these three hormones largely act at the intestines, kidneys, and bone to exert their regulatory effects[37].

In the dog, two pairs of parathyroid glands are located in the cranial cervical area[22]. The cells are arranged in nests and cords, delineated by a thin fibrovascular stroma. A rich capillary network is noted throughout the parathyroid gland and the predominant cells are groups or clusters of chief cells often arranged in small groups around a central capillary, giving a "pseudofollicular" appearance[40,41]. Histologically, these cells are cuboidal or polyhedral epithelial cells with lightly eosinophilic cytoplasm, but cytologically, cells with indistinct borders appear as free nuclei embedded in a background of lightly basophilic cytoplasm[22,41]. Other cells within the parathyroid gland include oxyphils, clear cells, and transitional oxyphil cells, with many believing these cells represent variations in the morphologic expression of chief cells[41]. With the help of transmission and electron microscopes, secretory granules are readily visible near the luminal border of variably active chief cells, which have well-developed organelles, aggregated endoplasmic reticulum, clustered ribosomes, and prominent Golgi apparatuses[40]. Secretory granules range from 100 to 300 micrometers and are more numerous in active chief cells than inactive chief cells[40]. The granules migrate to the periphery of the cell and fuse with the plasma membrane, extrude from the chief cell, detach, and enter circulation via nearby capillaries[40].

Neuroendocrine Cells

It was recognized in the late 1800's to 1930's that polypeptide hormone-producing cells, at the time called "clear cells", were widely dispersed not only within dedicated endocrine tissues, but also throughout a variety of nonendocrine tissues and organs, including

gastrointestinal tissue and skin[39,42, 43, 44]. This led to the concept of the endocrine system being more diffuse and dispersed throughout the body than previously realized, as well as the realization that the endocrine system could have local or systemic effects, leading to the term "diffuse neuroendocrine system" (DNES)[22,42,43,44]. These clear cells are now termed "neuroendocrine cells" (NECs). Neuroendocrine cells produce, contain, and store biologically active neuroamines and neuropeptides within membrane bound secretory granules[42,43,45,46]. These granules are released into the bloodstream to act as traditional neurotransmitters on cells at distant areas of the body and may also act on neighboring cells via paracrine signaling[44,46,47]. NECs share several cytochemical and ultrastructural characteristics and are also collectively referred to as amine precursor uptake and decarboxylase (APUD) cells[17,44,45]. These cells are apparently unrelated endocrine cells present in endocrine and nonendocrine tissues alike and are regularly present in all species[45,46]. Such cells include thyroid C cells that produce calcitonin, pituitary corticotrophs that produce ACTH, pituitary melanotrophs that produce melanocyte-stimulating hormone, and pancreatic β cells and α2 cells that produce insulin and glucagon, respectively[45].

It should be noted that the term neuroendocrine cell does not imply embryologic derivation from the neuroectoderm because the origin of NECs is variable. Rather it is meant to reflect the shared phenotypic characteristics these cells have with endocrine and neuronal cells[39,43,44,46]. Some do arise from the neuroectoderm, while others are epithelial and of endodermal origin, either dispersed throughout tissues or sometimes forming small structures within tissues. Other NECs are modified neurons that have no epithelial features and are neuroectodermal in origin, such as the adrenal medulla or parafollicular C cells of the thyroid[45,47].

Despite different origins, the majority of NECs are in fact epithelial, indicated by cytokeratin expression[46], and all NECs express some or all of the following markers: neuron specific elonase, synaptophysin, secretogranins, and chromogranins (referred to collectively as "neuroendocrine markers"), as well as the enzymes involved in their peptide hormone synthesis[47,48]. Of these, two commonly used neuroendocrine markers are chromogranin A (CgA) and synaptophysin (SYN)[49]. Chromogranin A is equivalent to parathyroid secretory protein 1 and is a commonly used marker for NECs in carcinomas arising in non-endocrine tissues[7,48,50,51]. Synaptophysin, primarily present in microvesicles, is a calcium-binding glycoprotein that is the most abundant integral membrane constituent of neuronal synaptic vesicles and is a common marker of NECs[43,48,52]. Synaptophysin has been demonstrated within neural-type and epithelial-type neuroendocrine neoplasms, with co-expression of neurofilament protein and cytokeratins, respectively[46]. Synaptophysin is more broadly expressed than CgA, as certain neurons, some pancreatic islet cells, and cells with sparse granules are negative for CgA but positive for SYN[43,46]. Thus, these two antibodies are commonly used as a complementary pair.

A few methods for determining neuroendocrine differentiation of cells have been developed, with silver impregnation techniques being one of the first and most useful. This technique utilizes the ability of endocrine cells to uptake and occasionally reduce silver ions; however, these techniques are very limited in poorly granulated NETs[48]. Advancement and growth of immunohistochemistry has revolutionized the ability to study these cell types, and as previously stated, multiple reliable markers of neuroendocrine differentiation are available[48].

As with any cell type within the body, NECs can undergo neoplastic transformation and a neuroendocrine tumor can form. Neuroendocrine tumors are a family of neoplasms that vary widely in their morphologic features, biologic behaviors, and functional capabilities. In humans,

NETs often contain mixed cell types and produce more than one hormone[48]. Ectopic hormone production is also possible, in which a NET secretes the hormonal product of another endocrine site[47]. Neuroendocrine cells may be dispersed as single cells or small cell groups within a tumor, such as within squamous cell carcinomas, adenocarcinomas, bronchopulmonary tumors, and certain carcinoids[43,53]. In these situations, they are only recognized after application of special immunohistochemical stains, and the presence of a minority of NECs within a tumor may have no significant impact[43]. Detection of specific NET hormones is possible but is sometimes challenging; poorly granulated NETs store too little product for detection, and some tumors produce abnormal forms of hormones that do not react with detection antibodies[46,48]. Given these difficulties, it is recommended that an array of markers be used in order to increase proper classification of tumor cells[48].

In human literature, there is debate about the cellular components of neuroendocrine tumors. There are "pure forms," which are NETs that are composed of neuroendocrine cells, and "mixed tumors" that are NETs admixed with non-neuroendocrine components[43,53,54]. Some authors require a tumor to have at least 20% non-neuroendocrine cells while others require an equal mix of neuroendocrine and non-neuroendocrine cells to be classified as a mixed tumor[43]. A study done by Sapino et al. looked at 50 NETs of the breast that were classified as such when ≥50% of cells showed positivity for neuroendocrine markers. In addition to the neuroendocrine markers, the authors looked within these tumors for the co-expression of gross cystic disease fluid protein-15 (GCDFP-15), an apocrine cell marker used for determining mammary differentiation. Interestingly, they found that both pure-neuroendocrine differentiation and neuroendocrine-apocrine differentiation may be found within breast tumors[55]. In this study, CgA was the most useful neuroendocrine marker, and its expression was significantly correlated with

the pure-neuroendocrine type tumors. Positivity for CgA generally correlates with secretory activity, with cells containing numerous and well-developed secretory granules exhibiting intense IHC staining[56]. On the other hand, paucigranular cells exhibit weak staining or may be completely nonreactive[43,48].

Neuroendocrine tumors can be generally grouped into epithelial and neural subtypes; this paper focuses on the epithelial subtype, which is defined as an epithelial neoplasm with predominant neuroendocrine differentiation[43,57]. Epithelial-type NETs can exhibit a range of histologic and cytologic features. There are three main histologic patterns: solid-type, made up of nests of neoplastic cells and occasional ribbon structure; rosette-type, containing mainly rosettes and glandular structures; and tubular-type, which have variably sized lumens lined by neoplastic cells[7,43]. Cells may be round, polyhedral, cuboidal, or spindle-shaped and vary in size from small to large. Granular eosinophilic or amphophilic cytoplasm is commonly noted and some cells may be vacuolated[43]. Nuclei may be round to oval and are sometimes located in the basal area of the cell. The nuclei can range from small in size, with hyperchromatic chromatin, to intermediate or large with coarsely granular chromatin. Nucleoli may be prominent but are not always noted[7,43].

Hypercalcemia and Hypercalcemia of Malignancy

Hypercalcemia can have transient, nonpathologic, or pathologic origins[33]. There are two main categories of pathologic hypercalcemia: parathyroid-dependent and parathyroid-independent[31]. Parathyroid-dependent causes include primary hyperparathyroidism and secondary nutritional or renal hyperparathyroidism. Parathyroid-independent causes include transient hypercalcemia, humoral hypercalcemia of malignancy (cancer-associated

hypercalcemia), idiopathic, and certain toxic, metabolic, skeletal, and granulomatous etiologies[31].

Hypercalcemia can manifest clinically in many ways due to impairment of the renal, gastrointestinal, and nervous systems[34,58]. Within the kidney there is decreased responsiveness to antidiuretic hormone at the distal tubule, decreased renal blood flow and GFR, and calcium salt deposition[58]. Polyuria, polydipsia, vomiting, dehydration, constipation, hypertension, weakness, shaking, bradycardia, and cardiac arrhythmias can also be seen[34,58].

Neoplasia, leading to paraneoplastic syndrome (PNS), is the most common cause of pathologic hypercalcemia in the dog[31]. Paraneoplastic syndromes are neoplasm-associated alterations in bodily structure, function, or both that occur distant to the tumor[58]. This type of hypercalcemia is termed humoral hypercalcemia of malignancy (HHoM) and when present, generally translates to a poor prognosis for the patient[26,31]. Lymphoma and AGASACA are considered the first and second most common neoplasms associated with HHoM[3,33,37,58,59]. Ten to forty percent of dogs with lymphoma show signs of hypercalcemia[60,61]. The numbers of hypercalcemic patients with AGASACA vary widely but fractions around 25% are reliably found throughout the literature[1,2,58]. Other neoplasms like multiple myeloma, thyroid carcinoma, bone tumors, thymoma, squamous cell carcinoma, and other carcinomas also, but less commonly, show a relationship with HHoM[34].

Neoplasms may cause hypercalcemia via production of PTHrP (see below), ectopic production of PTH, extensive lytic bone metastases, primary hyperparathyroidism, tumor-associated prostaglandin release, and other mechanisms[58,61,62,63]. Parathyroid hormone-related protein is a 160kDa protein synthesized broadly by many fetal and adult tissues and has been implicated as the cause of hypercalcemia in both lymphoma and AGASACA[31,58,64]. Parathyroid

hormone-related protein is structurally similar to PTH, which regulates serum calcium concentration, in that 8 of the first 13 amino acids are homologous to PTH. The next 21 amino acids, while not homologous to PTH, have been shown to effectively bind the PTH receptor, as the majority of differences in this region are conservative substitutions[29,34,65]. These structural similarities between PTH and PTHrP at the N-terminus of the protein are important because this is the functional portion of the molecule that effectively binds the PTH receptor[66]. In a study on PTHrP in human tumors, a rabbit polyclonal antiserum against PTHrP did not cross-react with PTH, indicating that the two peptides have distinct differences. However, PTHrP's ability to bind the PTH receptor suggests that the hypercalcemia seen in PTHrP-associated hypercalcemia of malignancy, such as in AGASACA, is due to the ability of PTHrP to function like PTH[58,67]. It has been shown that there are structural and antigenic similarities between human PTHrP and canine PTHrP and according to the National Center of Biotechnology Information (NCBI), human and canine PTHrP are 91.53% identical[68] (Figure 1).

Parathyroid hormone-related protein expressing cells have been identified within a variety of normal human and canine tissues[29,58,67]. Some of these tissues include keratinocytes within the basal layer of skin, epithelium of hair follicles, apocrine glands of the skin and anal sac, myoepithelial cells of the anal sac, thyroid gland C cells, mammary glands, bone, smooth and skeletal muscles, cardiac muscle, brain, and kidney[29,31,64,69]. The physiologic actions of PTHrP within these tissues are not completely known. Some proposed actions include regulation of the hair cycle and hair growth, regulation of keratinocyte proliferation and differentiation, stimulation and inhibition at the bone, stimulation of insulin secretion and somatic growth within pancreatic islet cells, mammary gland development, lactation, and release by smooth muscles in response to stretching[26,29,70].

The mechanism of humoral hypercalcemia of malignancy is variable across patients and tumors. Previous reports thought ectopic production of PTH by tumor cells was the main factor in generating HHoM; however, it is now believed that PTHrP is the primary factor, though the mechanism of increased PTHrP from neoplastic cells is not fully understood[34,64,67]. Local osteolysis is also considered an important mechanism in hypercalcemia of malignancy but less frequently results in hypercalcemia[34]. This occurs when there is a primary bone tumor or metastasis of a tumor to bone, which in turn activates osteoclasts to resorb bone[34]. For lymphoma, hypercalcemia in dogs may be due to HHoM or local resorption of bone due to bone metastasis, and studies have shown that neoplastic lymphocytes may generate hypercalcemia from mechanisms other than PTHrP production[9,63]. Kubota et al. showed that lymphoma cells from patients with and without hypercalcemia express PTHrP mRNA and thus can yield increases in circulating PTHrP. However, only patients with hypercalcemia had evidence of increased plasma PTHrP concentrations[64]. A study by Shallis et al. showed that the route of hypercalcemia differed between subtypes of lymphoma, and a broadly dominant mechanism as to the primary cause of lymphoma-associated hypercalcemia was not established.

AGASACA and Humoral Hypercalcemia of Malignancy

Parathyroid hormone-related protein is believed the primary mediator in humoral hypercalcemia of malignancy for AGASACA, as metastasis to bone is rare, and PTHrP has been detected within normal anal sac apocrine glands and AGASACA[9,34,67]. The physiologic significance of PTHrP in the normal anal sac apocrine gland cells is unknown, and the amount of PTHrP produced in health may not have a significant physiologic effect on the body, instead only working in a paracrine fashion when disease is not present[67]. There appears to be correlation

between immunohistochemical staining for PTHrP, serum calcium concentration, and serum PTHrP concentration in mouse models of AGASACA[62].

A direct link between tumor production of PTHrP has been shown in a mouse model using two tumor variants of a canine AGASACA. Grone et al. looked at two variants of a canine AGASACA in a mouse model and found that PTHrP production from the tumor was positively correlated with serum calcium concentration. One tumor variant was a well-demarcated and multilobulated AGASACA that had polygonal to cuboidal neoplastic cells arranged in acini, tubules, cords, and sheets. This tumor showed strong IHC staining for cytokeratin, produced high levels of PTHrP, and marked hypercalcemia was noted in mice with this tumor. The other AGASACA variant was a solid tumor made up of polygonal to spindle-shaped neoplastic cells arranged in sheets and cords supported by connective tissue stroma. Acini were rarely observed, PTHrP production was low, and mild increases in calcium were noted in comparison to control mice[62]. Possibly more significant is that Suzuki et al. showed that mixed-type tumors were more commonly associated with hypercalcemia, suggesting that the PTHrP expression of an AGASACA tumor may relate to the histologic pattern. Conversely, other studies indicate that production of PTHrP by a tumor does not necessarily lead to hypercalcemia[69].

Despite a correlation with hypercalcemia, not all patients with AGASACA become hypercalcemic. A study in humans identified PTHrP expression in 100% (34 samples) of squamous cell cancers but no patients were hypercalcemic[67]. This suggests that hypercalcemia occurs at a certain threshold of PTHrP production, which causes normal homeostatic control mechanisms to be lost; therefore, PTHrP expression by neoplastic cells should be correlated with serum calcium and PTHrP concentrations[67,68].

Tumor differentiation may also be a factor in the amount of PTHrP that is produced. Poorly differentiated tumors may be non-functional and secrete no or low amounts of hormone, such that no biologic activity occurs, while more differentiated tumors may have more functional capacity and cause an increase in PTHrP leading to systemic effects. Dogs with poorly differentiated tumors may survive longer and have a better prognosis than dogs with more differentiated tumors (i.e. have hypercalcemia)[2].

Anomalous AGASACA IHC Activity

It is previously established that the neoplastic cells that make up AGASACA are epithelial in origin. Interestingly, a few studies have determined that some normal anal sac apocrine gland cells, as well as neoplastic AGASACA cells, are positive for multiple neuroendocrine markers (chromogranin A and neuron-specific enolase)[5,6,7]. Synaptophysin has also been identified within AGASACAs but has not been noted within non-neoplastic apocrine cells[6]. Some non-NETs have also shown elevated CgA and neuroendocrine differentiation, which sometimes correlated with progression and poor prognosis[71]. There are rare reports of malignant apocrine carcinomas with neuroendocrine differentiation in human literature. Li et al. reported a tumor composed of cells that were round to polygonal with eosinophilic cytoplasm and a second population with abundant pale to foamy cytoplasm. Both cell types had enlarged nuclei with distinct nucleoli and were arranged in either a micronodular pattern or solid nests surrounded by densely fibroblastic stroma. Glandular or rosette-like structures (ductal lumina) were identified within the tumor. Immunohistochemistry revealed that tumor cells were positive for pan-cytokeratin, CK7, epithelial membrane antigen, and more than 80% were positive for synaptophysin and CgA[72]. If canine AGASACAs are found to consistently have significant

numbers of neuroendocrine positive cells, it may suggest a link to cancer-associated abnormalities caused by these tumors.

# CHAPTER 3

# MATERIALS AND METHODS

## Hypothesis

1. PTHrP expression is affected by tumor pattern of canine AGASACA
2. Tumors with greater neuroendocrine differentiation will express higher levels of PTHrP
3. Tumors that express higher levels of PTHrP will be associated with peripheral hypercalcemia

## Objectives

1. Validate PTHrP antibody for use in canine tissue samples
2. Evaluate the association between neuroendocrine differentiation and PTHrP antibody expression within canine AGASACA
3. Assess the relationship between peripheral calcium levels and PTHrP antibody expression within canine AGASACA
4. Assess the relationship between histologic tumor pattern and neuroendocrine differentiation in canine AGASACA
5. Assess the relationship between histologic tumor pattern and PTHrP antibody staining within canine AGASACA
6. Assess the relationship between peripheral calcium levels and histologic tumor patterns

Sample Selection

Study cases were selected from among archived tissues of canine anal gland tumors previously submitted to the Veterinary Diagnostic Laboratory (VDL) at the University of Illinois at Urbana-Champaign for histological diagnosis. The laboratory information system was searched for canine patient records between July 2008 and August 2019 in which the diagnosis of AGASACA was made histologically. Search terms included "AGASACA", "apocrine gland anal sac adenocarcinoma", "anal gland adenocarcinoma", and "anal sac adenocarcinoma". Clinical information was retrospectively obtained from medical records and the inclusion criteria consisted of a biochemical or ionized calcium measurement prior to surgical excision or euthanasia, confirmation of the previous histologic diagnosis of AGASACA, and sufficient archived tumor tissue (preserved, paraffin-embedded) to be further sectioned, stained, and evaluated. Archived H&E stained sections of the original tumors were briefly reviewed (SC, PR) to confirm agreement with the original tumor diagnosis. One hundred and forty-three cases were identified that fit the inclusion criteria. To ensure a representative group of tumor cases while balancing project costs, 120 of the 143 cases were included in this study. The decision to include 120 cases was based on recommendations from the American Society of Veterinary Clinical Pathologists for determination of *de novo* reference intervals[73].

Tissue Fixation, Processing, and Routine Staining

At the time of original submission, the tissue samples were fixed in 10% neutral buffered formalin. The tissues were processed routinely, embedded in paraffin wax and 5 µm sections were fixed onto slides. Slides were stained with H&E (process details below) using an automated stainer (Sakura) and a coverslip was applied.

1. Three changes of xylene at 3 minutes each
2. Two changes of 100% alcohol at 1 minute each
3. One minute in 95% alcohol
4. One minute in 70% alcohol
5. Rinse with deionized (DI) water for two minutes
6. Hematoxylin applied for 3 minutes and followed by a water rinse
7. Define – 30 seconds followed by water rinse
8. Blue Buffer applied for 1 minute and followed by a water rinse
9. One minute in 80% alcohol
10. Eosin applied for 1 minute
11. One minute in 95% alcohol
12. Three changes of 100% alcohol at 1 minute each
13. Three changes of xylene at 1 minute each

PTHrP Antibody Validation

Canine haired skin samples were used to validate the PTHrP antibody[67,69,74]. The antibody was optimized to identify the best performance. Dilutions and incubation times tested are as follows: 1:50 for 1 hour, 1:100 for 30 minutes, 1:100 for 1 hour, 1:100 for 2 hours, and 1:200 for 30 minutes. Each dilution was evaluated by SC and AS at low and high-power objectives. The 1:100 dilution for 2 hours was determined to give the best stain uptake with least background interference and was elected for use in the study.

IHC procedure for PTHrP antibody was as follows:

1. Antigen retrieval

i. Tissues were deparaffinized for at least 2 minutes in three changes of xylene, two changes of 100% ethanol, two changes of 95% ethanol, and a 70% ethanol solution

ii. Pretreatment with Diva Decloaker at 125°C for 30 seconds at 15-20 PSI and then 85°C for 10 seconds. Half the solution was decanted and refilled with tap water. This was repeated three times. The sample was then rinsed with tap water and two changes of TBS auto wash buffer.

2. Peroxidazed 1 was applied for 5 minutes
3. Blocked with background punisher for 10 minutes
4. Application with 1:100 dilution of PTHrP antibody for 2 hours
5. Secondary antibody was applied for 30 minutes, washed with buffer, and the secondary antibody was again applied for 30 minutes
6. 3,3′-Diaminobenzidine (DAB) was applied for 5 minutes
7. Counter-stained with cat hematoxylin for 5 minutes
8. Washed with buffer and deionized water

Immunohistochemistry Methods

Cytokeratin (epithelial marker), CgA (neuroendocrine marker), and SYN (neuroendocrine marker) were used to classify tumor cell lineage. IHC was performed using cytokeratin (CYK; Biocare Medical, pan-cytokeratin for CK8 and CK18; prediluted mouse monoclonal antibody), chromogranin A (ImmunoStar, rabbit polyclonal antibody diluted to 1:2000), synaptophysin (Biocare Medical, mouse monoclonal antibody, 1:100), and PTHrP antibody (ABCAM[74], rabbit polyclonal antibody, 1:100). Standard VDL control tissues were

used for each stain; sections of canine skin were used as positive controls for CYK, canine pancreas and adrenal tissue were used as positive controls for CgA, and canine pancreas and adrenal tissue were used as positive controls for SYN[6,7]. Sections of canine skin were used as positive controls for the PTHrP antibody[67,69]. The same tissues were used as negative controls, in which the primary antibody was omitted, and a universal polymer negative serum was placed on the slide.

IHC procedure for all samples was as follows:

1. Antigen retrieval
2. Peroxidazed 1 was applied for 5 minutes
3. Blocked with Background Punisher for 10 minutes
4. Application of primary antibody
    a. CYK, CgA, and SYN: 30 minutes
    b. PTHrP: 2 hours
5. Secondary antibody applied for 30 minutes, washed with buffer, and then applied again for 30 minutes
6. 3,3′-Diaminobenzidine (DAB) was applied for 5 minutes
7. Counter-stained with cat hematoxylin for 5 minutes
8. Washed with buffer and deionized water

Tumor Pattern

Histology sections (H&E and CYK) were evaluated at 12.5x, 20x, 100x, 200x, and rarely 400x and 500x (oil) magnification to clarify details regarding morphologic patterns. Tumors were classified as solid, rosette, tubular, and mixed (see below). Classification guidelines were

based on established histomorphologic descriptions of this tumor and from a study by Suzuki et al.[3,10,11,12] Solid tumors had sheets of round, normochromatic cells with a round, hyperchromatic nucleus, a prominent nucleolus, and a small amount of lightly eosinophilic cytoplasm. These cells were arranged in solid groups and nests surrounded by thin fibrovascular stroma and many solid tumors had small acinar structures formed around a central capillary or venule throughout. Rosette tumors consisted of cuboidal to columnar-shaped cells with a small to moderate amount of apical, lightly eosinophilic, cytoplasm and basilar-located rounded nuclei. Cells were arranged radially around variably sized circular lumen; these arrangements were commonly observed in groups and made up the majority of the tumor. Tubular tumors had large and variably sized lumina lined by rounded to cuboidal cells with a small amount of cytoplasm and round, hyperchromatic nuclei. Mixed tumors had clear and substantial evidence of 2 or more distinct patterns and were categorized accordingly (Solid + Rosette, SR; Solid + Tubular, ST; Rosette + Tubular, RT; and Solid + Rosette + Tubular, SRT).

Categorization of IHC Markers

IHC sections were microscopically evaluated at 12.5x, 20x, 100x, 200x, and rarely 400x, 500x (oil), and 1000x (oil) magnification. Higher magnifications were used to determine fine details and intracellular staining patterns. CYK expression was graded as positive or negative; positive samples were those in which greater than 50% of the cells displayed cytoplasmic cytokeratin reactivity. Cytoplasmic reactivity was defined as diffuse brown and slightly granular staining throughout the cytoplasm of cells.

Expression of neuroendocrine markers (CgA and SYN) and PTHrP were graded as negative (<5% of cells staining positive), weak (5-20% positive cells), moderate (21-50%

positive cells), and strong (>50% positive cells) based on subjective assessment by SC and PR at the previously mentioned microscopic magnifications. These grading percentages were based on a previous study by Suzuki et al. Evaluation of CgA and SYN rarely required magnification higher than 200x. PTHrP evaluation rarely required the use of greater than 500x (oil).

Estimated percentages of reactive cells for CgA, SYN, and PTHrP were determined at 12.5x, 40x, 100x, and 200x magnification. Tumor sections were divided into parts (1 part is equal to 1 microscopic field) depending on the size of the tumor. In each field the number of positive tumor cells were subjectively estimated. The estimated percentages of each field were added together and averaged by the number of fields counted (i.e. if twelve 200x fields were counted, these estimated percentages would be added together and divided by 12 to obtain the average estimated percentage of positive cells for the tumor). The number obtained was then used to categorize the tumor. If the estimated percent of positive cells was on a categorical cutoff (i.e. 4 or 5%; 20 or 21%; 50 or 51%) the process was repeated twice more on the tumor section and the three estimated percentages were added together, divided by three, and the resulting percent was used to categorize the tumor.

Statistical Analysis

Contingency tables were constructed using the data collected, including pattern types, presence of hypercalcemia, and PTHrP antibody expression. Depending on sample size for each category, data was analyzed using the Fisher's exact test and Pearson's chi-squared test to determine the relationship of hypercalcemia to histomorphologic tumor pattern or PTHrP antibody expression. A two-tailed t-test assuming unequal variances was performed to determine if age differed between male and female patients. A two-tailed t-test assuming equal variances

was performed to determine if age differed between normocalcemic and hypercalcemic patients. One way analysis of variance (ANOVA) was performed to evaluate the relationship between calcium values (mg/dL) and PTHrP antibody expression. Ionized calcium values in mmol/L were not analyzed as too few cases were available to determine significance within the study population. A $p<0.05$ was used to determine statistical significance. All data was analyzed in Microsoft Excel (Version 2102, Microsoft Office 365).

# CHAPTER 4

# RESULTS

## Sex and Age Predilection

Males and females made up 67.5% (81/120) and 32.5% (39/120) of the cases, respectively, with 98.3% (118/120) being neutered or spayed. A t-test assuming unequal variances failed to show a significant difference in the mean age of male and female patients diagnosed with AGASACA ($p=0.84$). A t-test assuming equal variances showed no significant difference in age of hypercalcemic and non-hypercalcemic patients ($p=0.82$).

The average age for the entire study population was 10.5 years, with a range of 5.4 to 18.5 years. The selected cases included 80 castrated males, 38 spayed females, 1 intact female, and 1 intact male. The age range for males was 5.4 years to 18.5 years with an average age of 10.5 years. The age range for females was 5.7 years to 14 years, with an average female age of 10.4 years. Breeds included 38 mixed breed dogs, 8 each of German shepherds and Labrador retrievers; 6 American cocker spaniels; 5 golden retrievers; 4 miniature schnauzers; 3 each of Australian shepherds, boxer dogs, cockapoos, collies, and miniature dachshunds; 2 each of American Staffordshire terriers, beagles, bichon frises, Cavalier King Charles spaniels, German shorthaired pointers, goldendoodles, Irish terriers, labradoodles, Rhodesian ridgebacks, and spitz; and one of each of the following: Airedale terrier, Alaskan husky, Australian cattle dog, basset hound, border collie, Brittany spaniel, Chesapeake Bay retriever, dachshund, English cocker spaniel, English springer spaniel, Irish setter, Norwegian elkhound, Portuguese water dog, Rottweiler, Shetland sheepdog, and silky terrier.

Histopathology, Tumor Patterns, and Hypercalcemia

The AGASACAs were categorized as solid (52/120; Figure 2), rosette (5/120; Figure 3), tubular (4/120; Figure 4), and mixed (59/120) tumors. Mixed tumors were further divided into specific subgroups of solid + rosette (SR; 24/120), solid + tubular (ST; 11/120), rosette + tubular (RT; 12/120), and solid + rosette + tubular (SRT; 12/120) (Table 1). Fisher's exact probability test and Pearson's chi-squared test showed no dependence of hypercalcemia on any of the seven histologic tumor patterns. There was also no significant dependence of hypercalcemia on the number of patterns present in one tumor (i.e. non-mixed tumors vs. mixed tumors; 1 pattern, $p=0.916$; 2 patterns, $p=0.746$; 3 patterns, $p=0.492$).

Immunohistochemistry

All 120 tumors were overwhelmingly positive for cytokeratin (>50% of the cells were positive) (Figure 5). There was mild to moderate background interference with chromogranin A (Figure 6), and most slides had a mild, light brown-orange hue. Interference staining was usually worse around the periphery of the tissue sections (Figure 7). When positive staining was observed, it appeared as fine, tan-brown granules in small aggregates, usually near the nucleus, and rarely diffusely spread throughout the cytoplasm. Rarely, tumors approached the 5% cutoff for CgA activity, but the overwhelming majority of samples showed far less than 5% CgA reactivity and all tumors were graded as negative. When present, non-neoplastic, normal-appearing glandular tissue occasionally showed positive reactivity for CgA (Figure 8). These cells were typically found in areas not affected by neoplastic cells, made up an exceedingly small portion of cells within the sample, and were not included when determining CgA reactivity for a given tumor.

All 120 tumors were graded as negative for synaptophysin. Positive SYN reactivity, noted as very fine, granular, diffuse, light brown, cytoplasmic staining, was exceedingly rare within samples. The vast majority of tumors showed nearly 0% reactivity for SYN (Figure 9). A single tumor showed positive SYN activity in approximately 3% of the neoplastic cells, in a focal area (Figure 10). No positive SYN activity was noted in non-neoplastic, normal-appearing anal sac apocrine glands.

As no tumors within the study population were categorized as positive for either chromogranin A or synaptophysin, no statistical testing was performed regarding neuroendocrine differentiation. The results for CgA and SYN activity are summarized in Table 1.

PTHrP and Hypercalcemia

Tumors were grouped by their subjective expression of PTHrP antibody. Of the 120 tumors, 62 (51.6%) were negative for PTHrP, 25 (20.8%) were weakly positive for PTHrP, nine (7.5%) were moderately positive for PTHrP, and 24 (20%) were strongly positive for PTHrP antibody expression (Figure 11; Table 1). When all tumors with positive PTHrP expression (weak + moderate + strong) were grouped together, Pearson's chi-squared test showed significant correlation between positive PTHrP expression and peripheral hypercalcemia ($p < 0.05$, $p=0.02$). Occasionally, PTHrP positive material was observed within acinar structures (Figure 12). This reactivity was not counted towards PTHrP categorization, as this staining was not cytoplasmic.

Thirty cases exhibited evidence of hypercalcemia (Table 1). The PTHrP expression within these tumors was: 10 (33.3%) negative, 8 (26.6%) weakly positive, 2 (6.6%) moderately positive, and 10 (33.3%) strongly positive for PTHrP antibody. One way analysis of variance

showed no significant correlation between peripheral calcium concentrations (mg/dL) and PTHrP activity.

Bilateral and Recurring AGASACA

In thirteen instances, bilateral AGASACA samples were submitted from a single patient, and these tumors were evaluated as separate tumors (26 total tumors; Table 2). Of these 13 cases, three had the same pattern and same PTHrP expression, one had the same pattern and different PTHrP expression, five had the same PTHrP expression but different patterns, and four had different patterns and different PTHrP expression.

There were three instances in which a dog had same site recurrence of previously excised AGASACA (Table 3). Case one had recurrence at 14 months. A change in histologic pattern was noted and PTHrP expression stayed the same; this patient was not hypercalcemic. Case two had recurrence at 19 months. A change in histologic pattern and PTHrP expression were noted; this patient was not hypercalcemic. Case three had recurrence at 14 months. Histomorphologic pattern was maintained in this patient, a change in PTHrP expression was noted, and hypercalcemia was present at both occasions of tumor removal.

# CHAPTER 5

# DISCUSSION

## Neuroendocrine Markers

Multidirectional differentiation, in which neuroendocrine tumors have evidence of non-neuroendocrine differentiation and non-neuroendocrine tumors contain subpopulations of neuroendocrine cells, has been shown to occur in humans[53]. Other studies have described neuroendocrine differentiation within AGASACA neoplasms, and it is a somewhat strange finding that, not only were all tissue sections negative for both neuroendocrine markers in this study, but that reactivity very rarely reached even 1% for either marker[7].

Problems with the chromogranin A marker were apparent from the beginning of this study. A variable but extensive degree of background interference was present within CgA tissue sections and because of this, extra time was taken to appropriately classify and evaluate these samples. Background interference was also observed in positive control samples but not negative controls. Multiple issues can arise that may lead to background staining, such as the presence of endogenous enzymes, endogenous biotin or lectins, secondary antibody cross-reactivity, and nonspecific antibody binding. However, Burchwalow et al. states that endogenous enzymes, endogenous biotin or lectin, and non-specific antibody binding are not really problems in the world of IHC today.

The background staining increased the threshold at which neoplastic cells were deemed truly positive. As a result, this decreased the sensitivity of the CgA marker and truly positive cases may have been missed. While this possibility should be seriously considered, it seems improbable that background interference caused significant alterations in the findings concerning

neuroendocrine differentiation, as all tissue sections were also steadily negative for synaptophysin immunostaining (a broader neuroendocrine marker)[46]. Studies that have found evidence of CgA positive cells within AGASACA neoplasms do indicate that the staining of neoplastic cells was variable, sometimes low in intensity, and CgA expression was located around the periphery of neoplastic cell nests[6,7]. While these studies demonstrate more convincing evidence of cytoplasmic staining for neuroendocrine markers, given the findings of this study, it should be considered that background interference may have played a role in the overinterpretation of neuroendocrine expression. Similar CgA and SYN antibodies, rabbit polyclonal and mouse monoclonal respectively, used in other studies were used in this study[6,7]. Higher concentrations of the antibodies for both CgA (1:2000) and SYN (1:100) were used in this study when compared to other studies, which used a 1:3000 dilution for CgA and a 1:500 dilution for SYN[6,7]. It is unclear, but considered unlikely, that the differences in antibody dilutions would have significantly impacted the results of this study, as positive control tissues displayed expression of the neuroendocrine markers. Furthermore, the author deems the minor differences in tissue processing between this study and other studies unlikely to have contributed to differences seen in neuroendocrine expression[6,7].

The significance of the single case with roughly 3% SYN positive cells is unknown. The staining of these cells was not correlated with CgA or PTHrP activity, as these cells and the neoplasm as a whole were negative for both. Due to cost, staining with a third available neuroendocrine marker, neuron specific enolase, was not pursued. Additionally, this marker lacks specificity and its expression within AGASACA tumors is variable in other studies[5,7,76].

The cytoplasmic granularity of the neoplastic cells is another consideration for the lack of neuroendocrine staining that was observed. Low granularity cells have been shown to be

negative for CgA[48]. In our study, it seems unlikely that all 120 AGASACA tumors would have a similar low granularity cellular morphology that hindered identification of neuroendocrine differentiation. Neuroendocrine cells use complex cellular pathways to store protein hormones within secretory granules[77]. Alterations in cell signaling, protein folding, and protein sorting within the neoplastic cells could have led to no or low CgA and SYN activity being noted. Classification systems in human medicine usually recognize two major groups of neuroendocrine carcinomas—well-differentiated and poorly-differentiated neuroendocrine carcinomas. Well-differentiated NETs express keratins and specific markers for neuroendocrine differentiation, such as CgA and SYN, but sensitivity and specificity of IHC markers can be reduced for poorly differentiated NETs[78]. A similar mechanism cannot be entirely excluded in the current study.

When looking at the totality of evidence in this study, the author concludes that these cells are not consistent with cells displaying neuroendocrine differentiation, as they showcased no neuroendocrine marker activity. Use of GCDFP-15, a marker of apocrine tissue, may have benefitted this study in terms of complete classification of the neoplastic cells[55]. Even though almost no neuroendocrine activity was noted within neoplastic cells, complete classification of these cells as apocrine origin would have been valuable.

PTHrP Expression

It is known that neoplastic cells can, but do not always, produce and secrete the same hormones of the cells from which they derive[79,80]. Positive PTHrP staining was found in 58 of the 120 neoplasms evaluated, but hypercalcemia was not noted in all these cases. This begs the question—how can both positive and negative PTHrP tumors cause hypercalcemia? A fully accurate and comprehensive answer to this question is difficult due to the myriad of factors at

play. Neoplasms leading to hypercalcemia may produce an abnormal variant of PTHrP that is undetectable with available antibodies. If the structure of the hormone was altered within the neoplastic cells, the polyclonal rabbit PTHrP antibody may not have been able to highlight PTHrP in some cases within this study. It is also possible that PTHrP is undetectable within neoplastic cells due to release and secretion of PTHrP at faster rates than it is synthesized or stored[78]. Lastly, other factors, such as interleukin-1, prostaglandins, and transforming growth factors, may have important roles in calcium regulation in patients with AGASACA but these factors were not evaluated in this study[79,81,82].

Tumor Patterns

As previously stated, no relationship was observed between hypercalcemia and tumor pattern. However, the small sample size for some of the tumor pattern groups could be considered as a limitation of this study. Five cases of the rosette pattern and four cases of the tubular pattern were observed. These small sample sizes decrease the power of statistical analysis and a larger sample size may have altered the results. This same issue arose with the analysis of PTHrP expression and tumor pattern, where too few cases were available for meaningful analysis.

Calcium Measurements

Not all hypercalcemic patients had tumors that were positive for PTHrP expression. PTHrP expression within AGASACA tumors correlated with hypercalcemia in this study, but this finding calls into question whether PTHrP is the only or even primary factor that contributes to hypercalcemia in the AGASACA patient. The retrospective nature of this study did not allow for

measurement of serum PTHrP, which would have been valuable in the assessment of PTHrP and may have been a better assessment of PTHrP expression. Correlating this study's findings to circulating concentrations of PTHrP may help build a stronger a link between calcium regulation and tumor PTHrP expression.

A limitation of this study was the inability to recover serum ionized calcium (iCa) measurements in all patients. Serum iCa evaluates the biologically active portion of calcium in the body, and total calcium is not able to accurately predict iCa[83]. This would be a useful addition to a prospective study design.

Bilateral and Recurring AGASACA

Too few cases of recurrent (n =3) and bilateral (n=13) AGASACA were present in this study to draw meaningful conclusions. However, it was interesting to note that time between surgical removal in the three recurrent cases of AGASACA was 14-19 months, with 15.7 months being the average. Other studies have reported recurrence times of 6-10 months[1,84]. Continued monitoring of patients after surgery can aid in earlier detection of recurrence and possibly longer survival times.

Currently, it is unclear whether metastasis of a primary AGASACA leads to bilateral disease or if two independent tumors arise within the anal sacs[20]. Cases have been documented where AGASACA appears within a previously "normal" anal sac after complete resection of AGASACA of the other anal gland[20]. It would be possible for a patient to present with unilateral AGASACA and have undetectable disease within the other anal sac. Further investigation, such as bilateral anal sacculectomy in dogs with unilateral disease, may be useful in additional characterization of this neoplasm. In this study, four bilateral AGASACA cases had tumors with

the same histologic pattern. Eight bilateral tumors had the same PTHrP activity, and three had the same histologic pattern and PTHrP activity. Differing histologic patterns in these samples may indicate these tumors formed separately from one another. However, many cellular changes occur during neoplastic transformation, which could lead to different phenotypes arising from the same neoplastic population of cells. Epithelial field carcinogenesis is a proposed explanation for the development of multiple primary or locally recurrent tumors, due to the presence of histologically and genetically abnormal tissue surrounding the original neoplasm[20]. This would be a fitting explanation for AGASACA if a concrete correlation between AGASACA development and recurrent anal sac impaction or infection could be demonstrated in future studies.

# CHAPTER 6

## CONCLUSIONS

The following can be concluded from the present study:

- Rabbit polyclonal PTHrP antibody is validated for use in canine skin and AGASACA to monitor cellular PTHrP activity.
- There is a significant correlation between PTHrP and hypercalcemia when ≥5% of apocrine gland anal sac adenocarcinoma cells express PTHrP activity.
- Apocrine gland anal sac adenocarcinoma is an epithelial tumor without neuroendocrine differentiation (chromogranin A and synaptophysin).
- The mean age of male and female dogs that develop AGASACA is roughly 10 years.

Future directions of this work may include detailed pursuit of the basic mechanism and role of PTHrP within the canine anal sac, as well as an intensive look into how malignant transformation alters the production and bioactivity of PTHrP. Overall, continued evaluation of tissue differentiation, PTHrP expression, and their roles in humoral hypercalcemia of malignancy may lead to earlier detection of disease, advancements in therapeutics used to treat dogs with AGASACA, and better patient outcomes.

CHAPTER 7

FIGURES AND TABLES

**Figure 1.**
**Comparison of human PTHrP (top) and canine PTHrP amino acids**

```
1    MQRRLVQQWS VAVFLLSYAV PSCGRSVEGL SRRLKRAVSE HQLLHDKGKS IQDLRRRFFL  60
1    MLRRLVQQWG VAVFLLSYSV PSCGRSVEEL GRRLKRAVSE HQLLHDKGKS IQDLRRRFFL  60

61   HHLIAEIHTA EIRATSEVSP NSKPSPNTKN HPVRFGSDDE GRYLTQETNKV ETYKEQPLK  120
61   HHLIAEIHTA EIRATSEVSP NSKPAPNTKN HPVRFGSDDE GRYLTQETNKV ETYKEQPLK  120

121  TPGKKKKGKP GKRKEQEKKK RRTRSAWLDS GVTGSGLEGD HLSDTSTTSL ELDSRRH  177
121  TPGKKKKGKP GKRKEQEKKK RRTRSAWLNS GVAESGLEGD HPYDISATSL ELNLRRH  177
```

*Red letters indicate differences between human and canine PTHrP amino acid sequences.

Homo sapien PTHrP: https://www.ncbi.nlm.nih.gov/protein/NP_945316.1

Canis lupus familiaris PTHrP: https://www.ncbi.nlm.nih.gov/protein/AAA82583.1

https://www.ncbi.nlm.nih.gov/protein

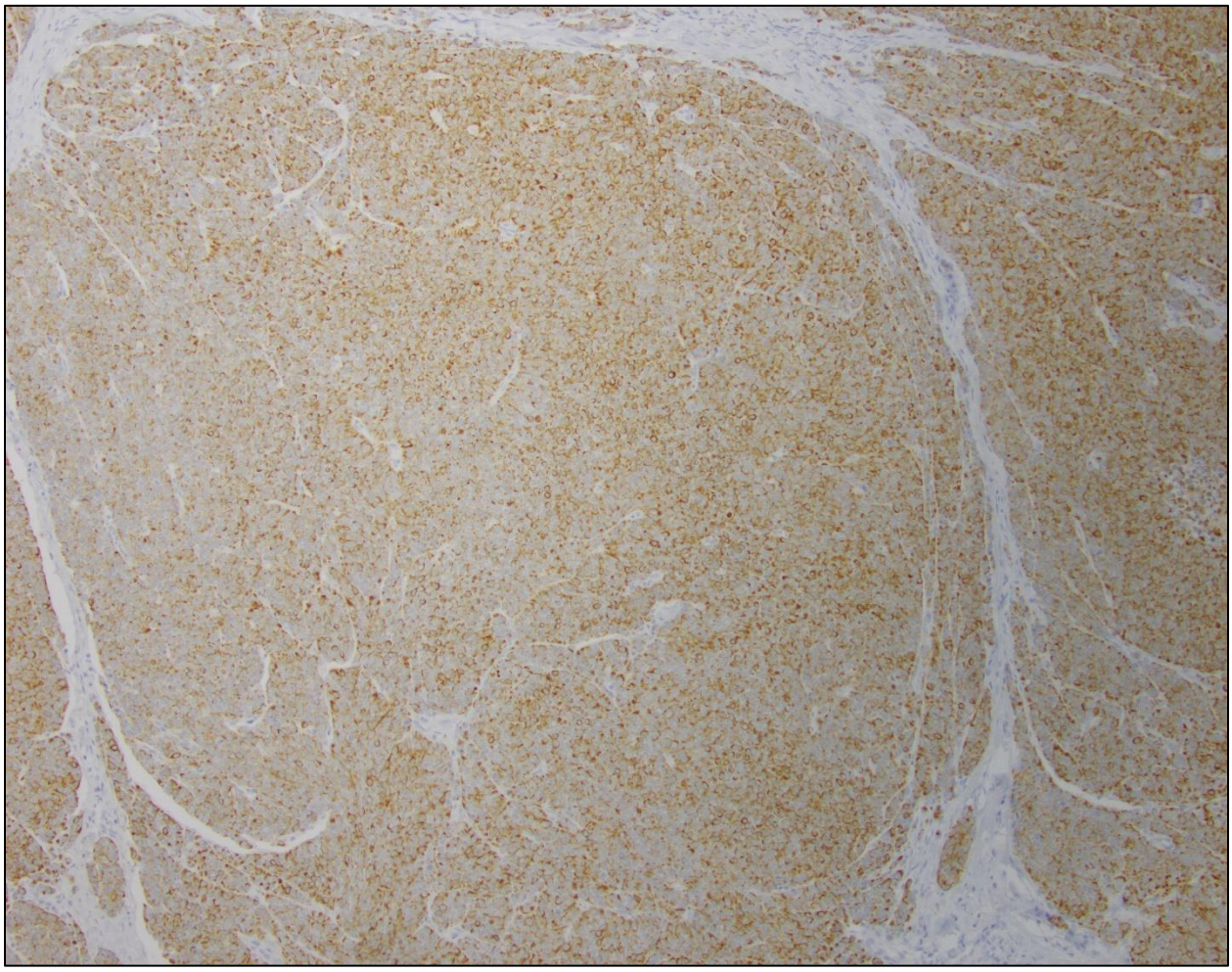

**Figure 2**. AGASACA with a solid pattern. Cytokeratin. x100

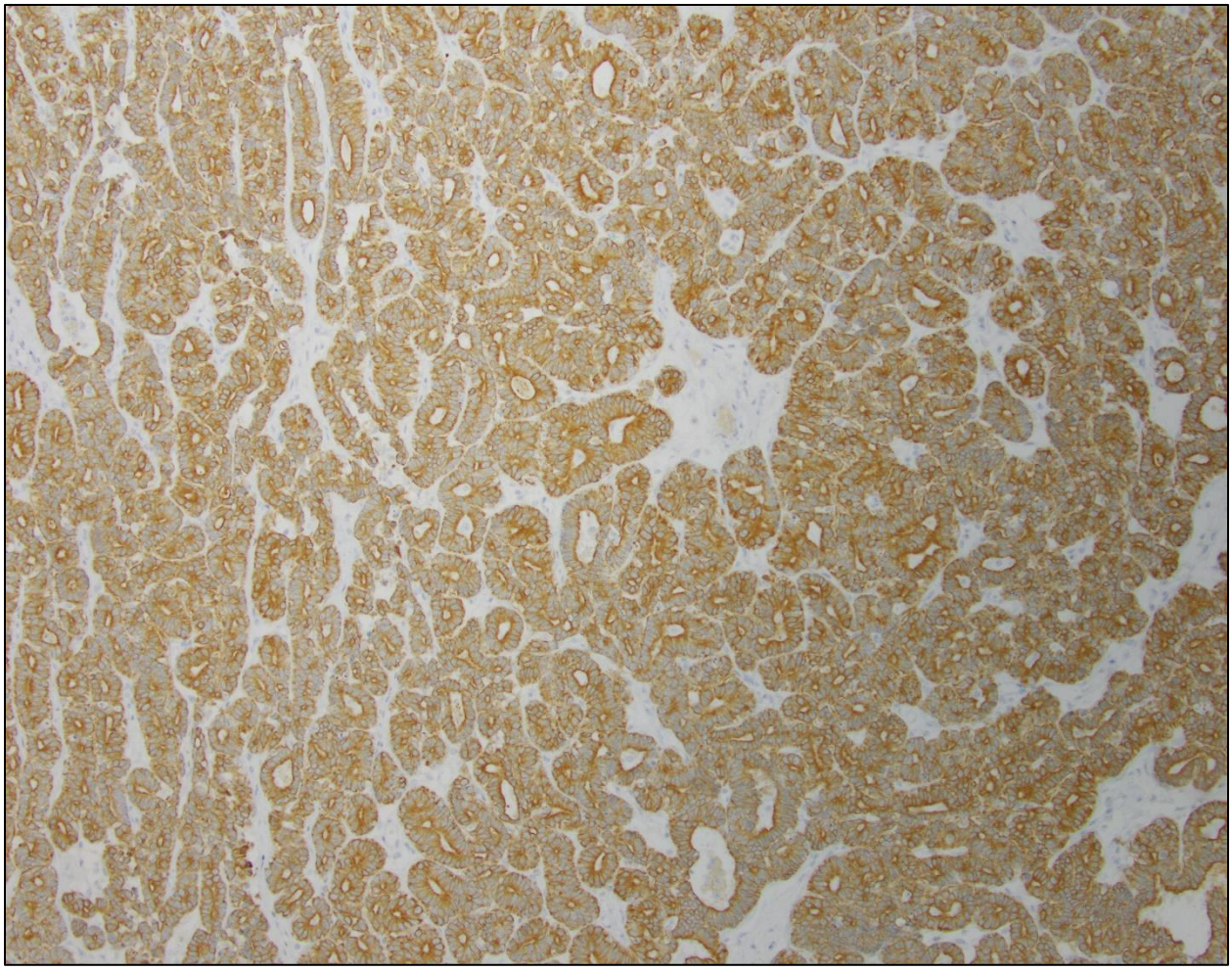

**Figure 3**. AGASACA showing a rosette pattern. Cytokeratin. x100

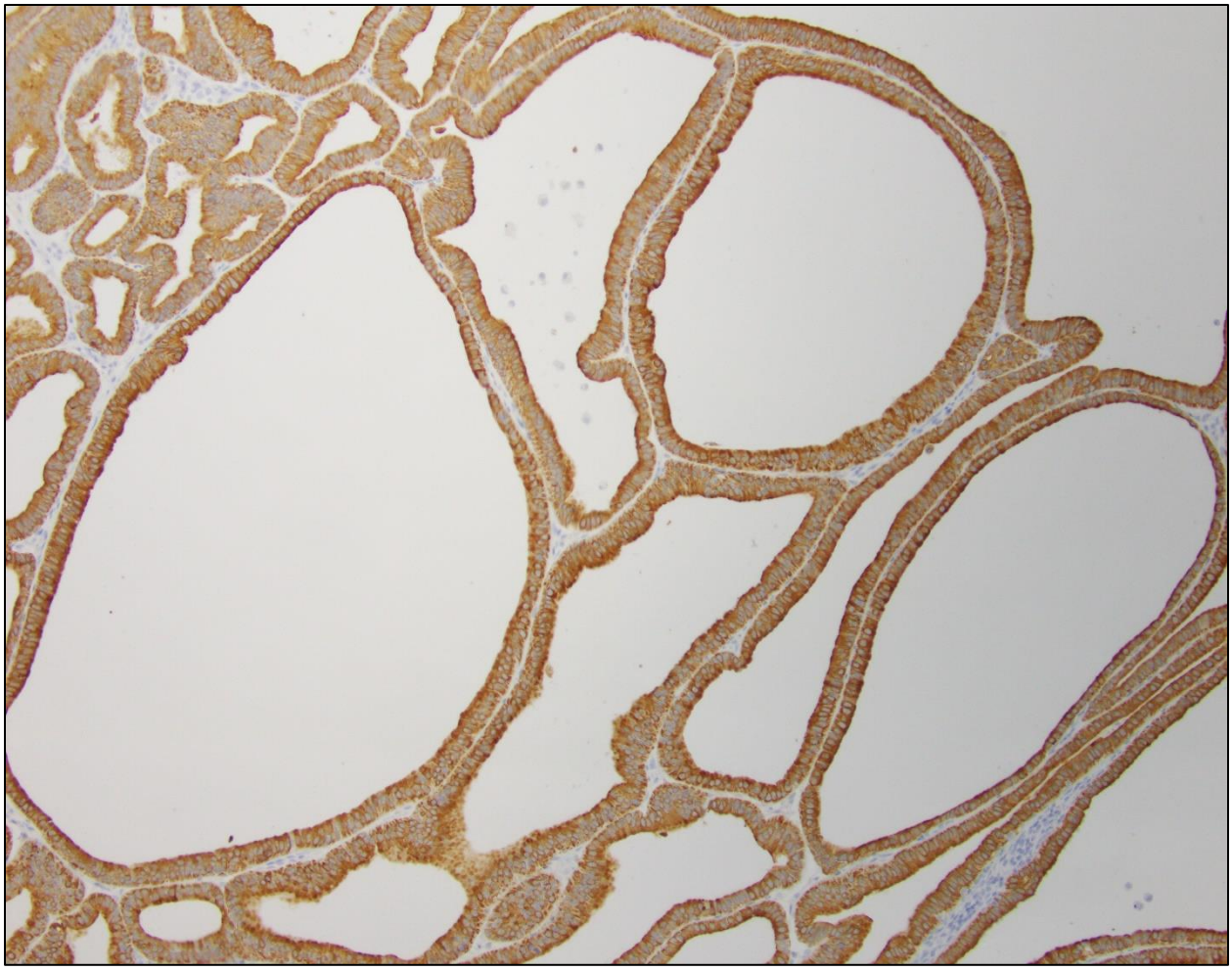

**Figure 4**. Tubular pattern within an AGASACA tumor. Cytokeratin. x100

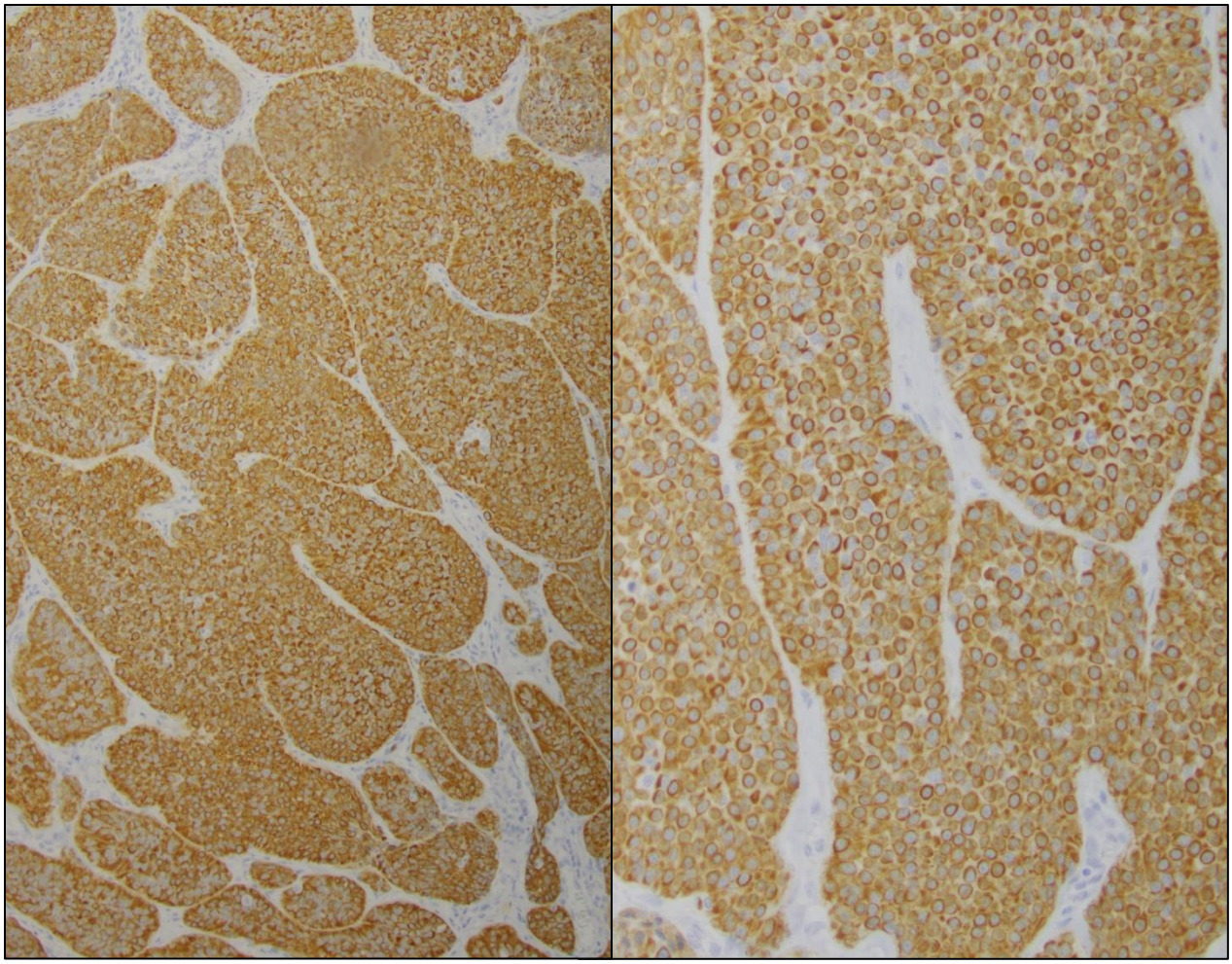

**Figure 5**. AGASACA with strong positive cytokeratin activity. Cytokeratin. **Left**: x100; **Right**: x200.

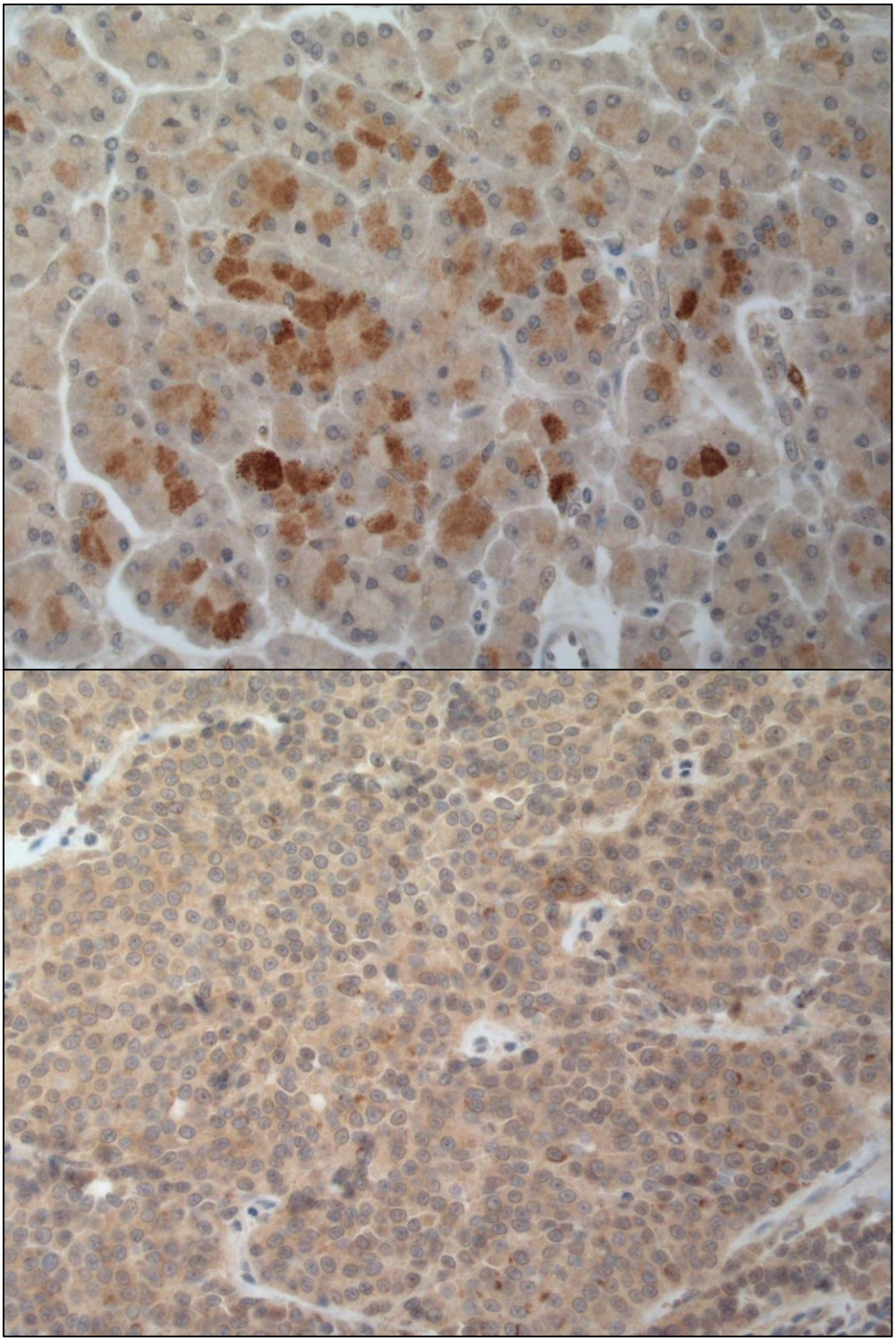

**Figure 6. Pancreas** (**top**): Positive CgA cells display moderately to deeply brown, cytoplasmic staining, with the most intense positivity in small aggregates near the nucleus. Light brown interference is noted within negative CgA cells. IHC. x500. **AGASACA** (**bottom**): Light brown interference is present throughout the sample, with very few cells showing true positive cytoplasmic reactivity for CgA. IHC. x500.

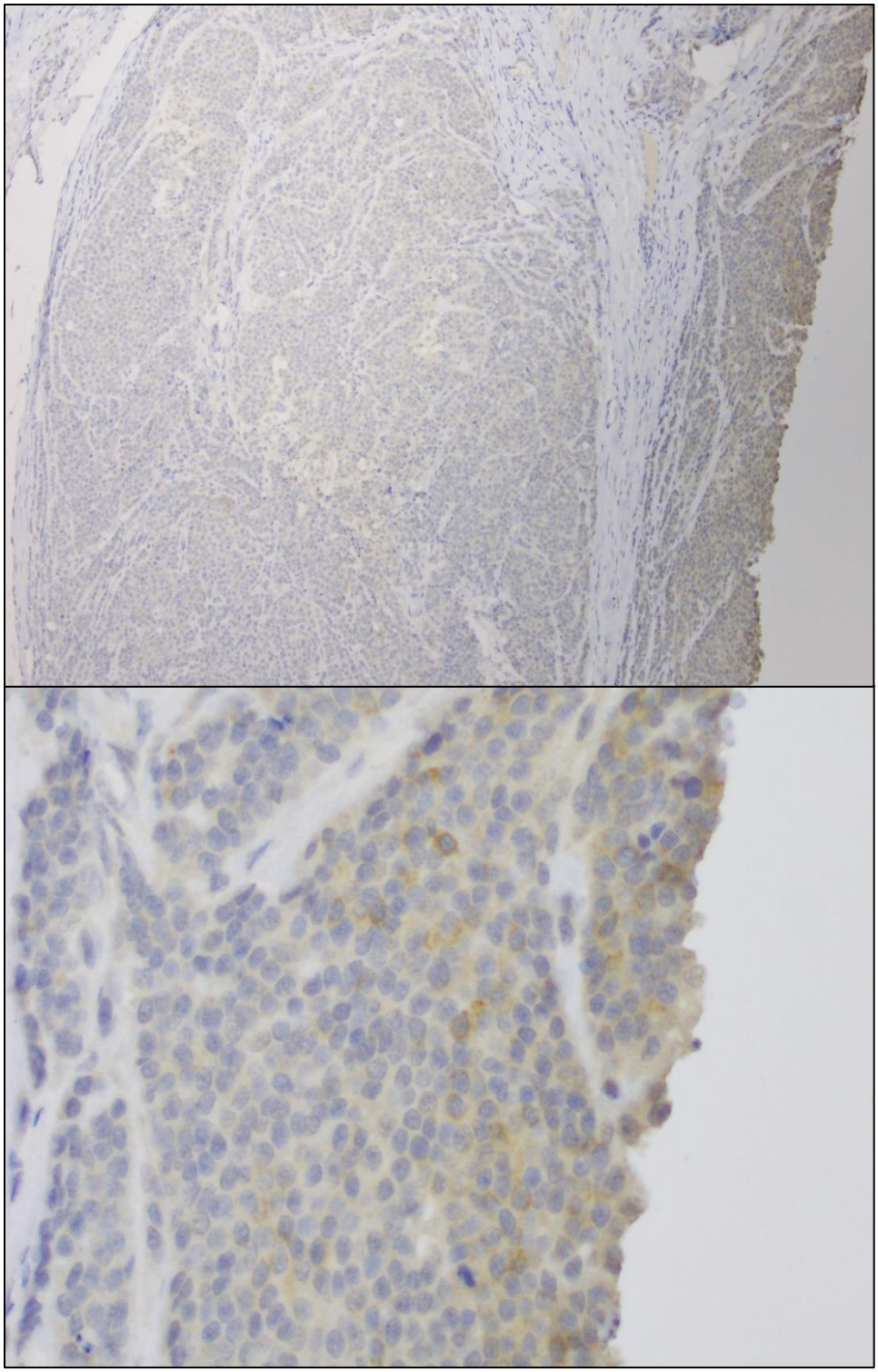

**Figure 7. Top**: An example of an AGASACA with worsening interference for CgA at the peripheral edge of the sample. IHC. x100. **Bottom**: Light brown, indistinct staining for CgA at the peripheral edge of an AGASACA tissue section. Rare cells contain positive CgA granules within their cytoplasm. IHC. x500.

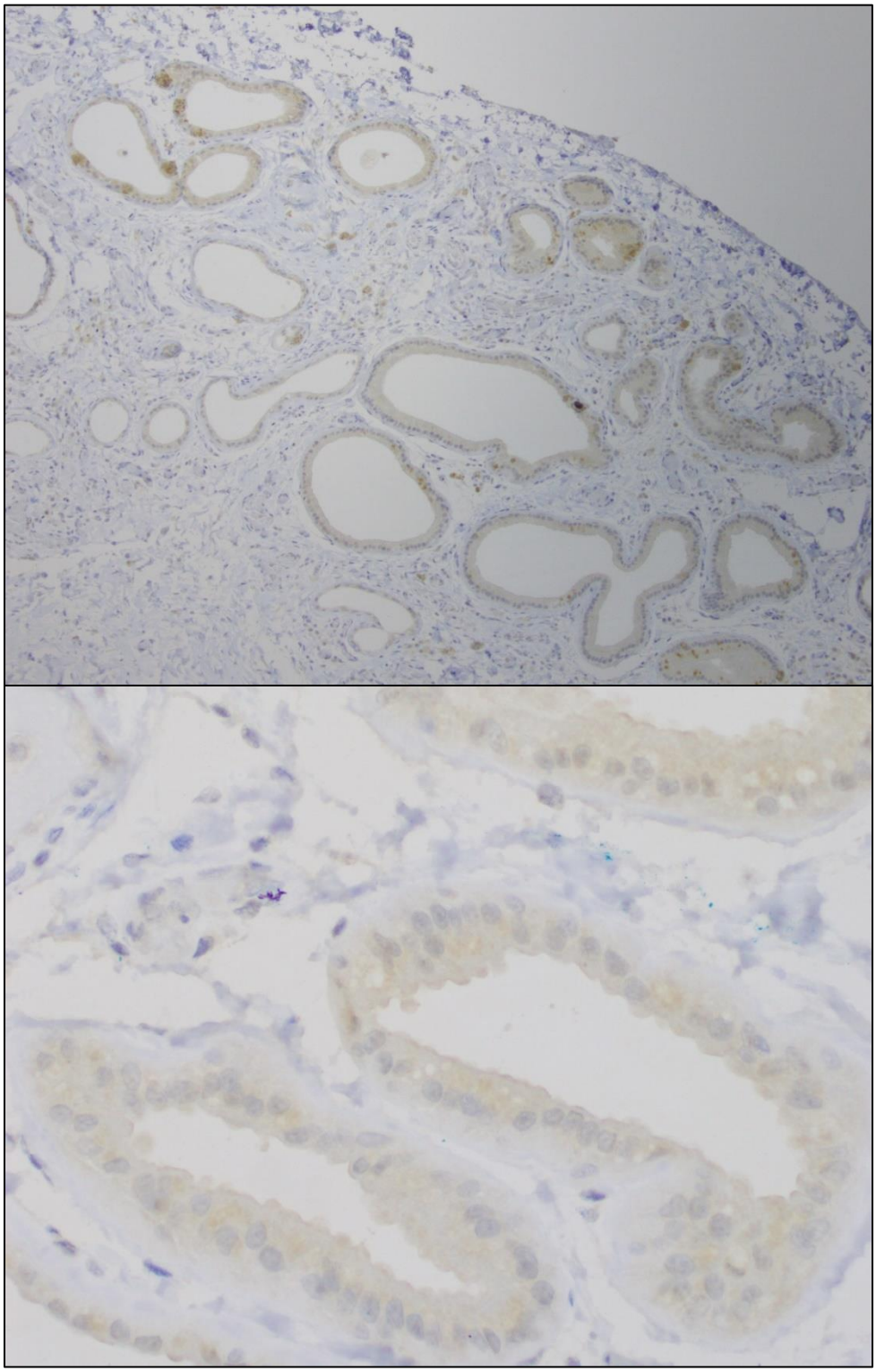

**Figure 8.** An area of non-neoplastic, apocrine gland cells. Low numbers of these cells are truly positive for CgA and light interference staining is noted. IHC. **Top**: x100. **Bottom**: x500.

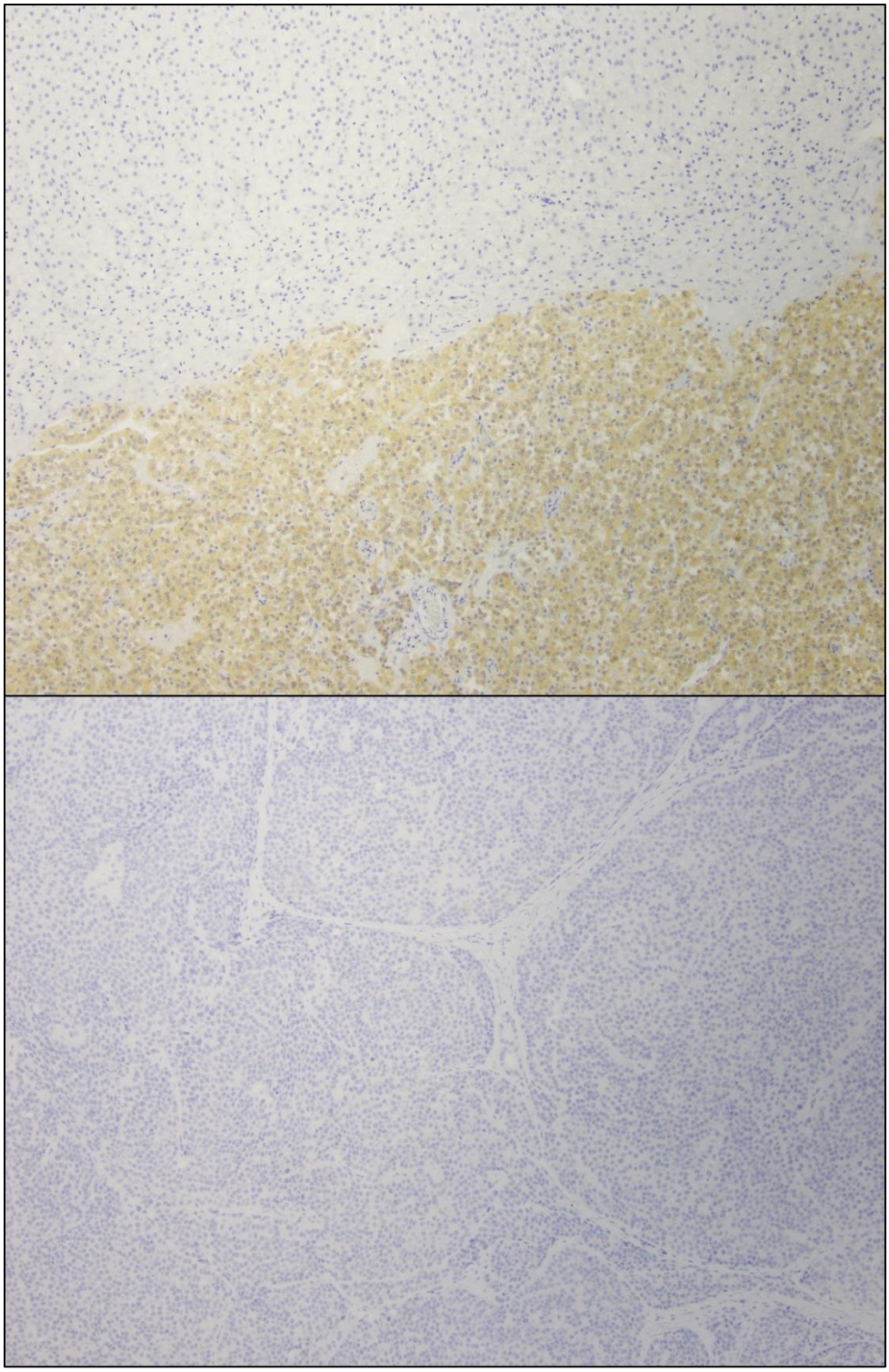

**Figure 9. Top**: SYN positive cells within the adrenal medulla. IHC. x100. **Bottom**: AGASACA with no SYN positive cells. IHC. x100.

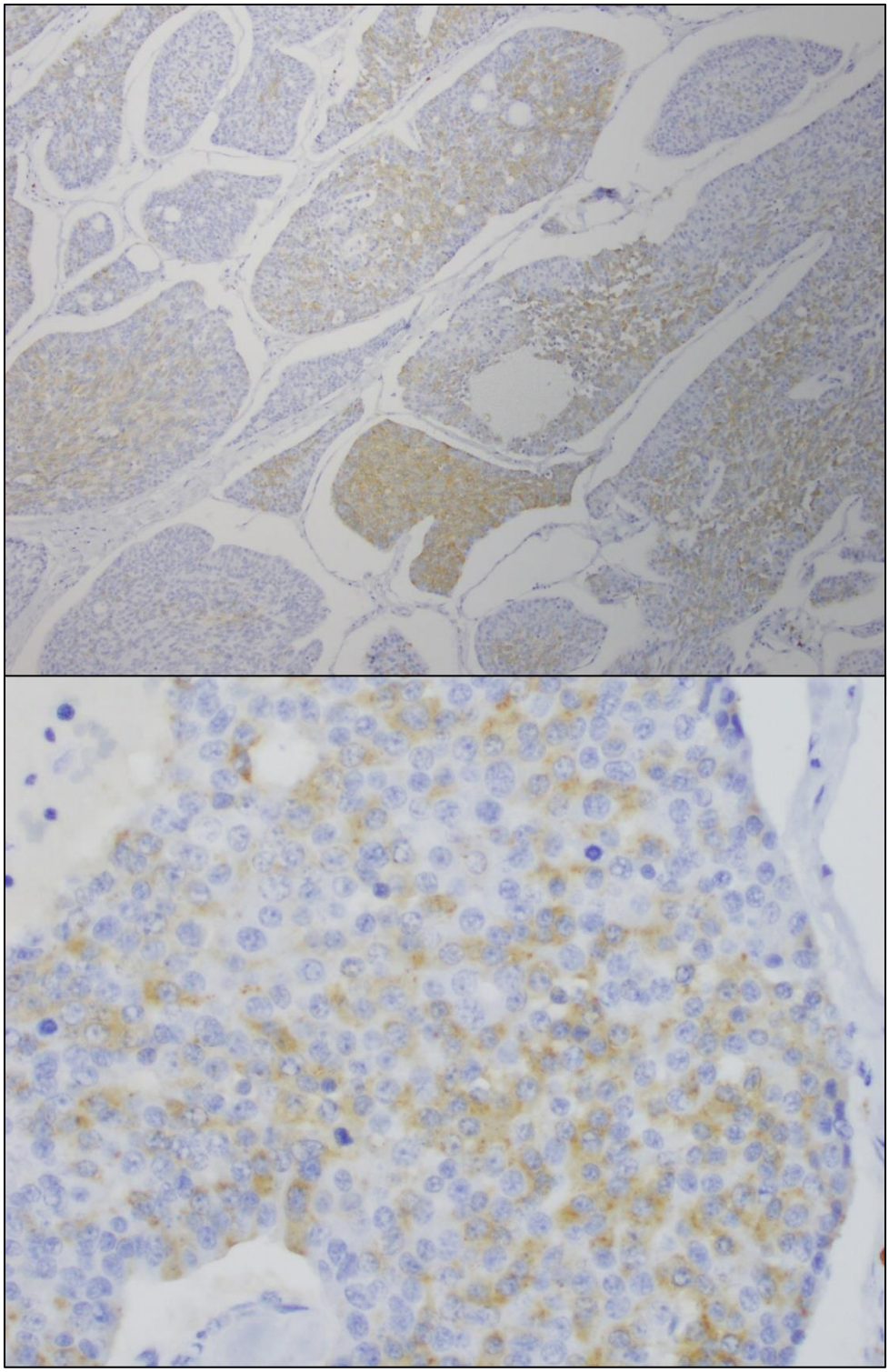

**Figure 10.** Small, focal area within a single AGASACA that contained SYN positive cells. A light brown, granular, cytoplasmic stain is observed. Overall, less than 5% of the neoplastic cells within this neoplasm showed positive SYN activity. IHC. **Top**: x100. **Bottom**: x500.

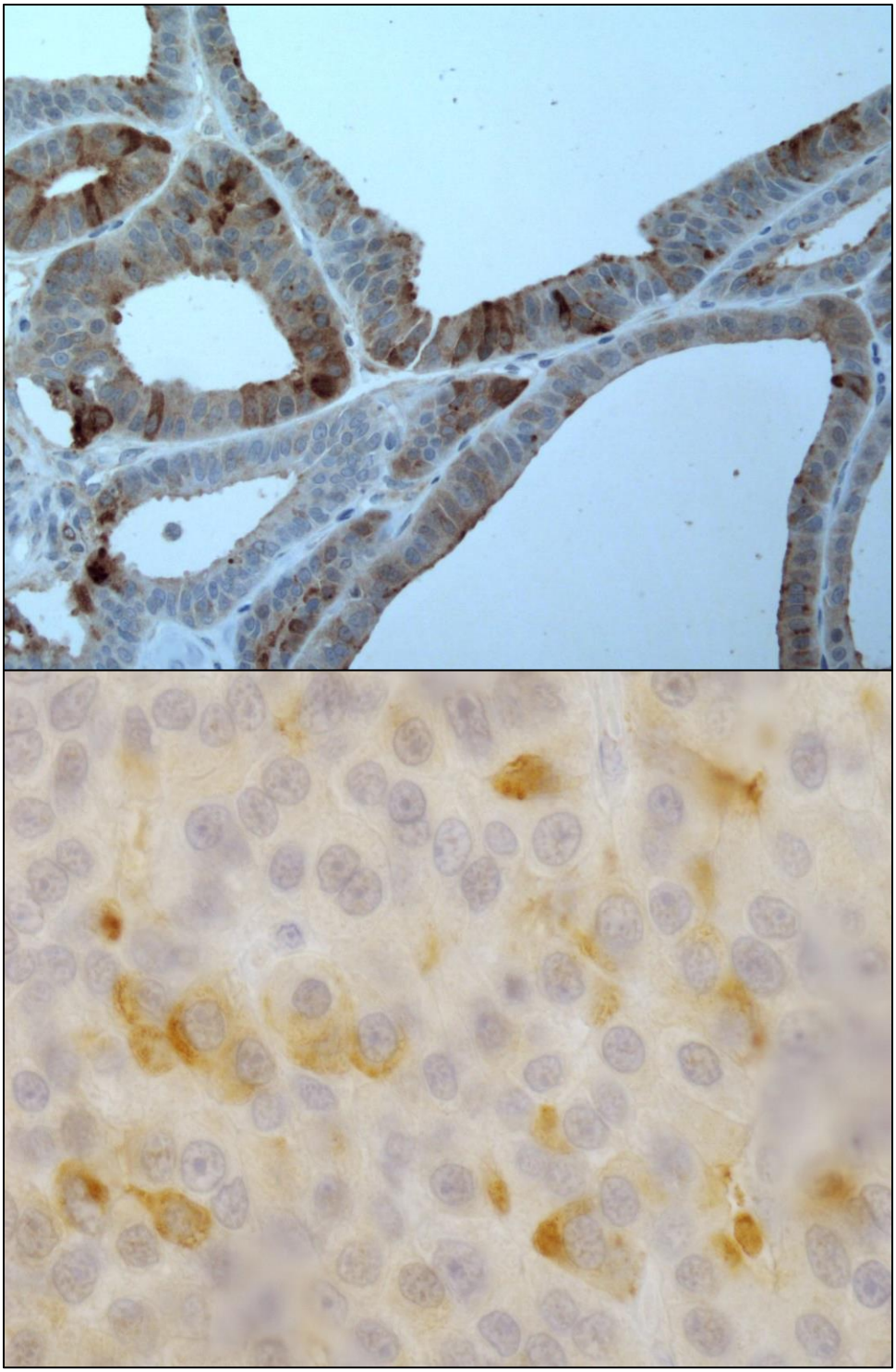

**Figure 11. Top:** Tubular pattern AGASACA with moderate PTHrP activity. Many cells in this image display variably brown cytoplasmic staining. PTHrP antibody. x500. **Bottom**: Mixed pattern AGASACA with weak PTHrP activity. Few cells display granular, golden brown, cytoplasmic staining. PTHrP antibody. x1000.

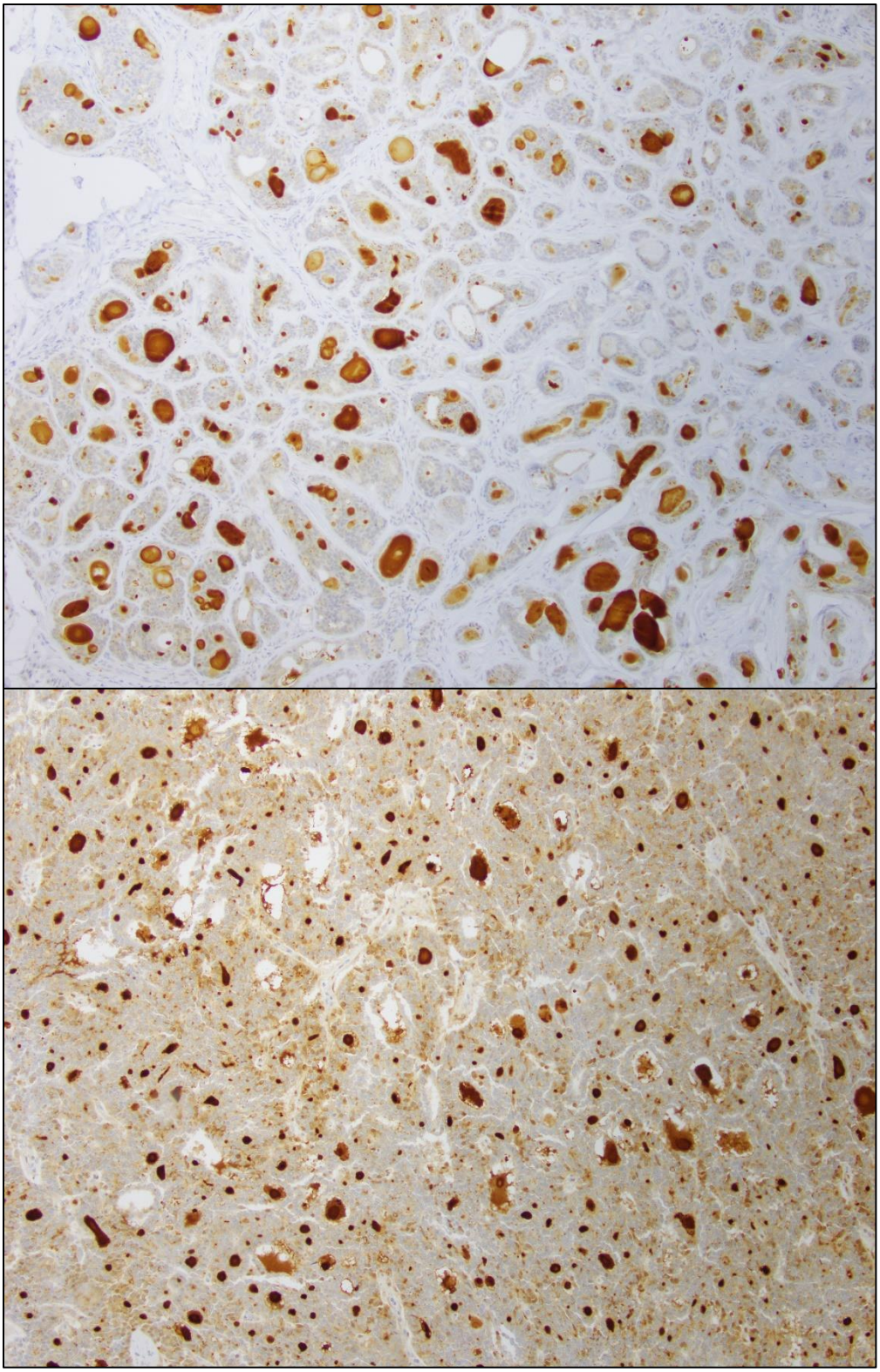

**Figure 12.** Two separate AGASACA tumors with moderate (top) and strong (bottom) PTHrP expression. Acinar structures contain PTHrP positive contents within their lumen. IHC. x100.

**Table 1.**

**Histologic pattern, staining characteristics, and serum calcium associated with AGASACAs**

| | Solid | Rosette | Tubular | Mixed | | | |
|---|---|---|---|---|---|---|---|
| | | | | S + R | S + T | R + T | S + R + T |
| **Epithelial Marker** | | | | | | | |
| Cytokeratin | | | | | | | |
| Positive | 52 (100%) | 5 (100%) | 4 (100%) | 24 (100%) | 11 (100%) | 12 (100%) | 12 (100%) |
| Negative | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| **Neuroendocrine markers** | | | | | | | |
| CGA | | | | | | | |
| Less than 5% | 52 (100%) | 5 (100%) | 4 (100%) | 24 (100%) | 11 (100%) | 12 (100%) | 12 (100%) |
| 5-20% | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| 21-50% | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Greater than 50% | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| SYN | | | | | | | |
| Less than 5% | 52 (100%) | 5 (100%) | 4 (100%) | 24 (100%) | 11 (100%) | 12 (100%) | 12 (100%) |
| 5-20% | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| 21-50% | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Greater than 50% | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| **PTHrP activity** | | | | | | | |
| Less than 5% | 32 (61.5%) | 0 (0%) | 1 (25%) | 16 (66.7%) | 5 (45.5%) | 2 (16.66%) | 6 (50%) |
| 5-20% | 13 (25%) | 1 (20%) | 0 (0%) | 5 (20.8%) | 3 (27.3%) | 2 (16.66%) | 1 (8.3%) |
| 21-50% | 0 (0%) | 2 (40%) | 1 (25%) | 1 (4.2%) | 1 (9%) | 2 (16.66%) | 2 (16.7%) |
| Greater than 50% | 7 (13.5%) | 2 (40%) | 2 (50%) | 2 (8.3%) | 2 (18.2%) | 6 (50%) | 3 (25%) |
| **Serum calcium concentration** | | | | | | | |
| Hypercalcemic | 12 (23.1%) | 3 (60%) | 0 (0%) | 7 (29.1%) | 2 (18.8%) | 2 (16.6%) | 4 (33.3%) |
| Not hypercalcemic | 40 (76.9%) | 2 (40%) | 4 (100%) | 17 (70.8%) | 9 (81.8%) | 10 (83.3%) | 8 (66.6%) |

S + R, solid and rosette patterns
S + T, solid and tubular patterns
R + T, rosette and tubular patterns
S + R + T, solid, rosette, and tubular patterns

**Table 2.**
**Comparison of bilateral AGASACAs**

| | Histologic pattern | PTHrP activity |
|---|---|---|
| Tumor 1.1 | Solid | **Negative** |
| Tumor 1.2 | Mixed; S + R | **Negative** |
| Tumor 2.1 | Mixed; S + T | **Negative** |
| Tumor 2.2 | Solid | **Negative** |
| Tumor 3.1 | Mixed; S + R | **Negative** |
| Tumor 3.2 | Solid | **Negative** |
| Tumor 4.1 | Solid | **Negative** |
| Tumor 4.2 | Mixed; S + R | **Negative** |
| Tumor 5.1 | Mixed; S + T | **Weak** |
| Tumor 5.2 | Mixed; S + R | **Weak** |
| Tumor 6.1 | Mixed; S + R | Weak |
| Tumor 6.2 | Mixed; R + T | Strong |
| Tumor 7.1 | Mixed; S + R + T | Negative |
| Tumor 7.2 | Solid | Weak |
| Tumor 8.1 | **Solid** | **Weak** |
| Tumor 8.2 | **Solid** | **Weak** |
| Tumor 9.1 | Solid | Negative |
| Tumor 9.2 | Mixed; S + R | Weak |
| Tumor 10.1 | **Mixed; S + R** | **Negative** |
| Tumor 10.2 | **Mixed; S + R** | **Negative** |
| Tumor 11.1 | Mixed; S + T | Negative |
| Tumor 11.2 | Mixed; R + T | Moderate |
| Tumor 12.1 | **Solid** | **Negative** |
| Tumor 12.2 | **Solid** | **Negative** |
| Tumor 13.1 | **Mixed; S + T** | Strong |
| Tumor 13.2 | **Mixed; S + T** | Moderate |

Bolded results indicate the two tumors share those characteristics.

**Table 3.**
**Cases of recurrent AGASACA**

| | Histologic Pattern | PTHrP Expression | Hypercalcemia | Time of recurrence* |
|---|---|---|---|---|
| **Case 1** | | | | |
| First occurrence | Mixed; S + R + T | Negative | No | 14 months |
| Second occurrence | Mixed; S + R | Negative | No | |
| **Case 2** | | | | |
| First occurrence | Mixed; S + R | Negative | No | 19 months |
| Second occurrence | Solid | Weak | No | |
| **Case 3** | | | | |
| First occurrence | Solid | Weak | Yes | 14 months |
| Second occurrence | Solid | Strong | Yes | |

* Time between the first and second surgical removal

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